

5-1-2013

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Vaibhav V. Joshi

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Comparison of indigenous and bio-augmented pentachlorophenol (PCP) degrading
bacteria for remediation of PCP in contaminated groundwater

By

Vaibhav V. Joshi

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Forest Products
in the Department of Forest Products

Mississippi State, Mississippi

May 2013

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Comparison of indigenous and bio-augmented pentachlorophenol (PCP) degrading
bacteria for remediation of PCP in contaminated groundwater

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The objective was to compare pentachlorophenol (PCP) degradation in contaminated groundwater by indigenous and bio-augmented (*Sphingobium chlorophenolicum* and *Burkholderia cepacia*) PCP degrading bacteria. Indigenous bacteria were identified by cloning and sequencing of 16S rDNA fragments while PCP concentrations were determined by GC-ECD. Gene expression for PCP degrading enzymes: chlorophenol 4-monooxygenase (TftD, *B. cepacia*) and pentachlorophenol-4-monooxygenase (*pcpB*, *S. chlorophenolicum*), was determined by RT-PCR. *B. cepacia*, a PCP degrading bacteria was identified as dominant indigenous bacteria. PCP concentrations correlated negatively with PCP tolerant bacteria and relative fold gene expression in treatments with air-sparging (phase2) compared to without air-sparging (phase1). PCP concentrations decreased and TftD or *pcpB* expressions were higher in treatments inoculated with *B. cepacia* (49%, 10.7 fold) or *S. chlorophenolicum* (32%, 7 fold), respectively, than un-inoculated (indigenous) or mixed culture inoculated

treatments. Thus bio-augmentation of indigenous bacteria with *B. cepacia* or *S. chlorophenolicum* resulted in more PCP degradation than indigenous bacteria.

DEDICATION

I would like to dedicate this work to my father Venkatesh P. Joshi, my mother Sandhya V. Joshi, my brother Swapnil V. Joshi, friends and “Shree Swami Samarth” Pariwar. Their support and encouragement helped me through difficult times.

ACKNOWLEDGEMENTS

I would like to acknowledge and express my sincere gratitude to my adviser Dr. M. Lynn Prewitt for her guidance, encouragement and support in all aspects of my research. Without her help this work could never have been completed. I wish to thank my graduate committee members Dr. Hamid Borazjani, Dr. Din Pow Ma, Dr. Kenneth O. Willeford and Dr. Dragica Jeremic for sharing their expertise, opinions and valuable time. Thanks are also due to Dr. Susan Diehl for providing laboratory equipment for my research. I would like to thank Mr. Min Lee for his invaluable help in this project. Finally I would like to acknowledge research funding from the Department of Forest Products, Mississippi State University and Mississippi Water Resources Research Institute.

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CHAPTER I

INTRODUCTION

Chlorophenolic compounds are used in a wide range of industrial and agricultural applications such as pesticides, paints, pulp bleaching, leather tanning and wood preservatives, which can also make them common environmental contaminants. The most common sources of these contaminants are the improper storage and disposal (Vallecillo et al. 1999). Toxicity of these chlorophenol compounds is directly proportional to their degree of chlorination (Karn et al. 2010a). Among these, pentachlorophenol (PCP) is a five chlorine containing aromatic phenolic compound, which makes it not only a very efficient wood and leather preservative, but also a toxic contaminant in the environment (Kao et al. 2004). Widespread use of PCP for more than sixty years has resulted in groundwater contamination, which is a very serious health and environmental issue (Langwaldt 1998). Prolonged exposure to PCP may lead to an increased incidence of non-Hodgkin's lymphoma, multiple myeloma and cancer in humans, specifically in people working with PCP (Cooper and Jones, 2008). Therefore PCP is listed by the U.S. Environmental Protection Agency (US EPA 1987) as one of the priority pollutants and its use in residential construction is restricted. Thus it is very crucial and urgent to effectively remediate PCP and its impurities causing contamination in the environment. Moreover PCP is very resistant to degradation due to the presence of the stable aromatic

ring with a high chloride content, which makes it a persistent environmental contaminant (Copley 2000).

Chemical and physical treatments for remediation of PCP contaminated sites have disadvantages including high cost and production of secondary pollutants. In contrast, biological treatments are cheaper and environmentally friendly without formation of any hazardous by-products. PCP can act as a source of carbon and energy to some microorganisms, which also facilitates its degradation. Many bacterial species have been reported to actively degrade PCP such as *Burkholderia cepacia*, *Pseudomonas sp.*, *Sphingobium chlorophenolicum*, *Arthobacter sp.*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus thuringensis* and *Acinobacter sp.* ISTPCP-3 (He et al. 2008, Karn et al. 2010a and Sharma et al. 2009). Among these bacteria, *B. cepacia* and *S. chlorophenolicum* are extensively studied with respect to their PCP degradation mechanism (Yang et al. 2006).

Genes responsible for production and control of enzymes in PCP degradation pathway have been identified, sequenced and located within the genome of *B. cepacia* and *S. chlorophenolicum* (Cai and Xun 2002). Conversion of PCP to tetrachloro-*p*-benzoquinone (TCBQ), the first step in the degradation pathway, has been determined to be the rate limiting step in PCP degradation pathway. Thus expression of gene encoding chlorophenol 4-monooxygenase (TftD, *B. cepacia*) or pentachlorophenol-4-monooxygenase (*pcpB*, *S. chlorophenolicum*) which regulates this first step is crucial in bacterial degradation of PCP (Crawford et al. 2007).

The aforementioned studies are based on single organisms under laboratory conditions. However in the environment there are many microorganisms present at sites of PCP contamination and only a few studies have investigated the microbial community

and their activity in PCP contaminated groundwater. The main objective of this work is to compare indigenous bacteria to bio-augmented known PCP degrading bacteria for remediation of PCP contaminated groundwater. The hypothesis for this study is that an indigenous microbial community bio-augmented with known PCP degrading bacteria degrades more PCP than an indigenous microbial community in PCP contaminated groundwater. Therefore the specific objectives were:

- To identify an indigenous bacterial community in the PCP contaminated groundwater
- To screen four microbial cultures: indigenous bacteria, indigenous plus 2 known PCP degrading bacteria (*B. cepacia* and *S. chlorophenolicum*) and indigenous plus a mixed culture of *B. cepacia* and *S. chlorophenolicum*
- To compare the RNA expression of genes coding for PCP degrading enzymes: chlorophenol 4-monooxygenase (*B. cepacia*) and pentachlorophenol-4-monooxygenase (*S. chlorophenolicum*), during PCP degradation by these four microbial consortiums.

CHAPTER II

LITERATURE REVIEW

Wood preservatives: historical perspective

Wood preservatives increase the service life of many wood products by providing protection from degradation caused by microorganisms, insects, marine borers, fire and weathering. Wood preservatives date back to 2000 B.C. when natural oils were used to protect wood from decay (Barnes 2002). Subsequently, coal tar derivatives and salt solutions have been used for preserving wood. Creosote, a coal tar byproduct was patented in 1836 by Franz Moll and its use in pressure treatment impregnation processes patented by John Bethell in 1838 greatly impacted wood preservation (Freeman et al. 2003). The Bethell or full cell process was the first method to use pressure treatment of wood and currently serves as the basis for most modern wood treating processes for utility poles, landscape timbers, railroad ties and other wood products. In addition to creosote other wood preservatives are classified as oil borne organic compounds (pentachlorophenol, PCP) and water borne inorganic compounds (chromated copper arsenates, CCA) (Barnes 2002).

Effectiveness of chemical preservatives depend on the toxicity of the compound, method of application, extent of penetration, and retention in treated wood. These chemical preservatives are highly toxic to wood destroying organisms, penetrate into the wood and remains in the pressure treated wood for years.

In the United States, wood preservatives are managed and regulated by the Environmental Protection Agency (EPA), under the Federal Insecticide, Fungicide and Rodenticide Act in order to protect the environment. The EPA regulates air and water quality standards, limits chemical discharge into the environment, and oversees certification, registration, remediation and penalties for noncompliance of toxic wood preservatives. Many wood treatment plants currently follow the regulations of EPA for storage, processing and discharge of generated wastes. However prior to the establishment of these regulations, improper handling of many wood preservatives for many years has resulted in soil and groundwater contamination. Currently the EPA lists 179 PCP contaminated sites in the superfund database and 3 within the state of Mississippi (Comprehensive, Environmental, Response, Compensation and Liability Information System, CERCLIS, 2012).

Pentachlorophenol (PCP): chemical characteristics and uses

PCP is a five chlorine containing phenolic compound (Figure 2.1) that is commonly used as a pesticide, in pulp bleaching, leather tanning and as wood preservative. In 1929, L. P. Curtin patented the use of chlorine derivatives of coal-tar acids having molecular weights higher than cresols (methyl phenols, MW 108) for wood preservation. The production of PCP as a wood preservative first began in the 1930s on an experimental basis. Because PCP was a low cost, highly effective and universally applicable chemical, it was later produced commercially in 1936 by Dow and Monsanto Chemical Companies (Cedar 1984).

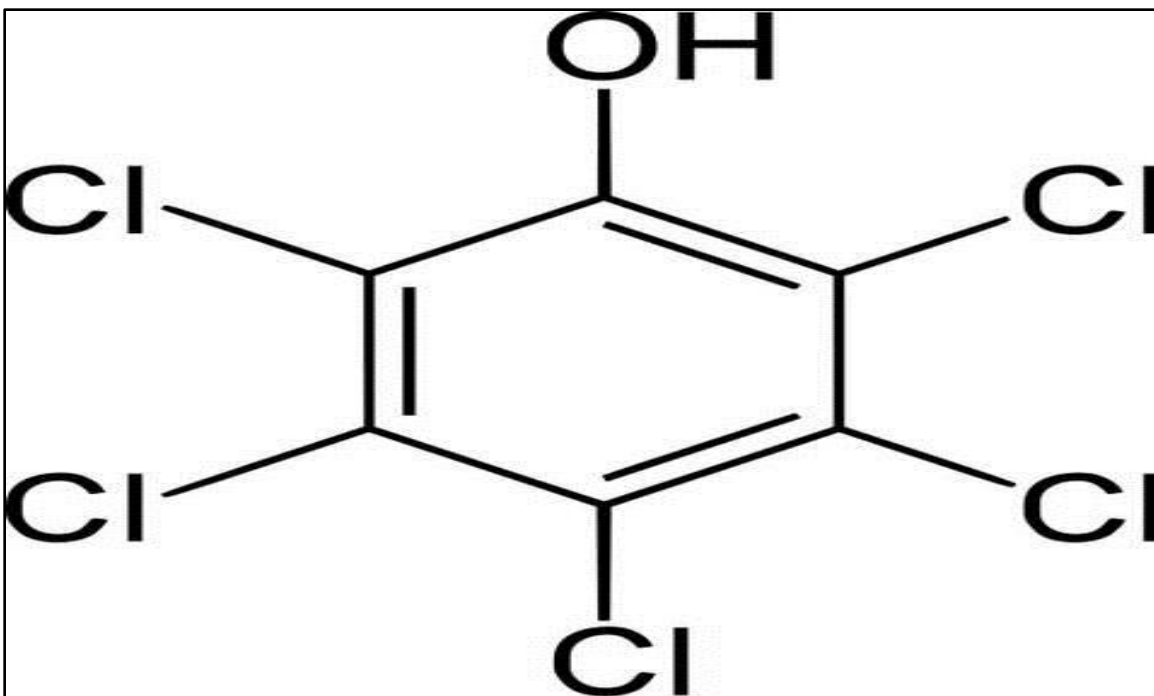


Figure 2.1 Structure of pentachlorophenol

Note: Reference: <http://en.wikipedia.org/wiki/Pentachlorophenol>

In 1977, approximately 50 million pounds of PCP were produced annually in the United States (Fisher 1991). PCP is usually applied in a number of treatment methods as liquid formulations (5% solution) in carrier oils such as P-9 oil, No. 2 fuel oil, kerosene or mineral spirits (US EPA 2004). After pressure treatment, PCP penetrates into wood and binds to the middle lamella between cell walls and is retained in the treated products under most environmental conditions (Crosby 1981).

Technical grade PCP used for wood preservation contains a number of toxic impurities (approximately 10%) such as chlorinated dibenzodioxans, hexachlorobenzene, dibenzofurans and cyclohexadienes. These compounds vary from the mildly toxic octachlorodibenzodioxin to very toxic hexachlorobenzene and dioxin HxCDD (Crosby 1981, Tondeur et al. 2010). Due to its toxic effect on the environment, commercial use of

PCP was restricted in 1987 by US EPA. The use of PCP was further restricted in 1990 when PCP was proven to be a potential human carcinogen (US EPA 2008) and currently is used only in the pressure treatment of lumber and utility poles. For these reasons, as of 2009 the commercial production of PCP had decreased to approximately 16 million pounds (Vander 2010).

Environmental impact of pentachlorophenol

Widespread use of chlorophenolic compounds in industrial and agricultural applications potentially makes them common environmental contaminants. Toxicity of these compounds is directly proportional to their degree of chlorination (Karn et al. 2010a). Among these, PCP, a priority pollutant and a B2 probable human carcinogen is listed by the US EPA because of its toxic effects on the environment (Kao et al. 2004). PCP has been found in significant levels in soil, surface water and groundwater especially around sawmills and wood treatment facilities. It has been reported that 96.5% of PCP contamination is found in soil, 2.5% in water, 1% in air and less than 1% in suspended sediments and organisms in the aquatic environments (Hattemer-Frey et al. 1989). PCP can persist in soil from 14 days to 5 years or longer depending on microorganisms present in the soil and environmental conditions (US EPA 1978). The principle reason for environmental contamination by PCP is caused by improperly disposed wood treatment wastes (Lee et al. 2006). Treatment of wood results in penetration of PCP into wood but immediately after treatment unbound PCP may bleed out of the wood in a liquid or crystal form and can be easily washed away from the surface of treated wood into the environment. Thus not only improper storage of PCP, but also improper storage of

freshly treated wood products can be a potential source of PCP contamination near wood treatment facilities.

Major breakdown products of PCP by organisms found in the environment under aerobic and anaerobic conditions include tetrachloro-p-benzoquinone; 2, 4, 6-trichlorophenol and 2, 6-dichlorophenol which are also toxic. Pentachloroanisole is also found in the environment due to methylation of PCP by bacteria and is more toxic than PCP (US EPA 2004).

Because PCP is a relatively volatile compound (vapor pressure= 0.00017 mm Hg at 20 °C) (Warren et al. 1982), it also has been detected at low levels of 0.0005 to 0.01 parts per billion (ppb) in the indoor air of wood treatment facilities as well as pressure treated log homes brushed with PCP (ATSDR 1999). PCP deposited in soil can migrate from soil to subsurface and groundwater which leads to further contamination. Aqueous solubility of PCP is an important factor in ecotoxicology studies because it indicates bio-availability for the microbial degradation. PCP is only slightly soluble in water (10 to 20 mg/kg, 25 °C), is pH dependent and its solubility increases with an increase in pH (Arcand et al. 1995). In 1974, the Safe Drinking Water Act passed which requires EPA to determine the level of contaminants in drinking water at which no adverse health effects occur (maximum contaminant level MCL). EPA has set an enforceable regulation for PCP with MCL value of 1 ppb.

Human exposure to PCP

Human exposure to PCP occurs in indoor or outdoor air, through drinking water and food. PCP can therefore enter the human body via inhalation and ingestion of contaminated air, food or water and by skin contact with treated wood.

Exposure to PCP is more significant in people working in wood preservation facilities, sawmills and other workplaces involving PCP treated wood (US EPA 1990). Although most people are not occupationally exposed to PCP, 85% of urine samples taken from the general U.S. population contained PCP with concentrations of 6.3 ppb or greater. For workers who were occupationally exposed to PCP, these levels increased to 37 ppm (McLean et al. 2008). People living in industrialized areas show increased levels of PCP from 1 to 100 ppb in urine samples, indicating greater exposure (Dougherty 1978). PCP also has been detected in human blood, fat tissue and breast milk (Ryan 1985, Noven 1988). People living in PCP treated log homes or in direct contact with PCP treated wooden products show higher levels of PCP in blood (average of 420 ppb) compared to the general population (average of 40 ppb). Children can have approximately 1.8 times more PCP in blood than adults as they have higher metabolic rates and thus inhale more PCP (Cline et al. 1989). Approximately 99.9% of all PCP contamination in the environment is found in soil, the food chain (especially fruits, vegetables, grains) and meat products which are significant sources of human exposure to PCP (Figure 2.2). PCP residues ranging from 1 to 100 ppb were found in selected food items such as powdered dry milk, soft drinks, bread, cereals, noodles, rice, sugar and wheat due to the storage in PCP treated wooden containers. Animal products like beef, pork, chicken and milk are also found to be contaminated with PCP due to direct or indirect contact through feed mixed with PCP treated wood products such as wood shavings or sawdust used as a litter.

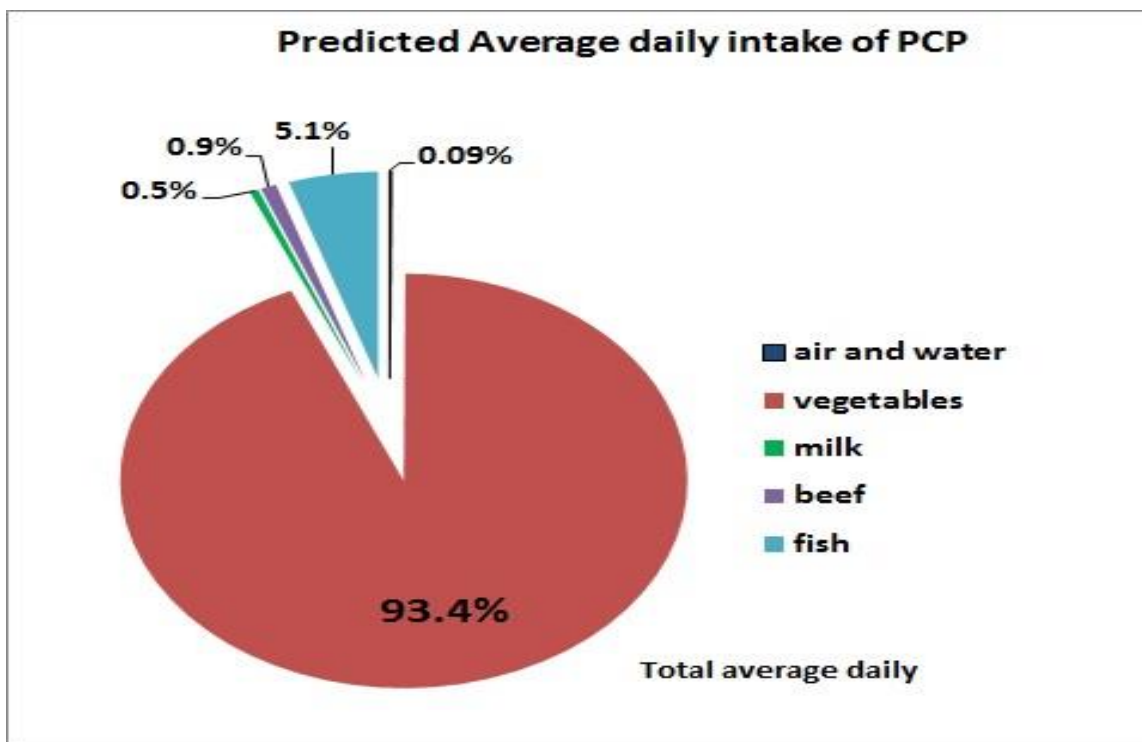


Figure 2.2 Predicted average daily intake of pentachlorophenol from various sources.

Note: Reference: Fisher 1991.

In case of acute exposure, PCP is rapidly excreted from the body, mainly through urine as unchanged PCP or as PCP glucuronide while in case of chronic exposures, the elimination half-life can be up to 20 days. Longer elimination time can be attributed to the large fraction of absorbed PCP which is protein bound and widely distributed in body tissues including kidney, heart and adrenal glands (Kalman and Horstman 1983). The principle toxicological mechanism of PCP in the body is via enhanced cellular oxidative metabolism resulting from the uncoupling of oxidative phosphorylation, which leads to excessive rise in body temperature (hyperthermia) due to heat produced during uncoupling (Shen et al. 2005).

The reference dose (RfD) which can be used to estimate hazardous health effects, for PCP is 0.03 mg/kg body weight per day (US EPA 1999). The most common symptoms of acute toxicity of PCP include irritation to mucous membranes, dermatitis, profuse sweating, increased respiration and heart rate, fever, abdominal pain, nausea, weakness, dizziness and anorexia. Chronic toxicity symptoms include aplastic anemia, immunological effects due to accumulation of PCP in liver, kidney, brain, spleen and fatty tissues (ATSDR 1999, US EPA 1999). PCP and its contaminant hexachlorodibenzo-p-dioxin (HxCDD) may affect the reproductive system resulting in birth defects, abortions, infertility and skeletal anomalies. Ingestion of water containing 3.0 ppb repeatedly over time may result in increased chances of developing cancer (ATSDR 1999). Exposure to PCP may lead to Non-Hodgkin's lymphoma, multiple myeloma, kidney cancers, soft tissue sarcoma, acute leukemia, hepatocellular adenoma and carcinoma especially in those working with PCP for many years (Dermers et al. 2006, Cooper and Jones 2008). Although there is no clear cut determination of the lethal dose of PCP, consistent levels of PCP in human serum greater than 1 part per million (ppm) can lead to potential health hazards (Gary et al. 1985).

Remediation of environmental contaminants

Remediation of contaminants in the environment refers to the removal or degradation of the contaminants from environmental media such as water, soil or air. Remediation methods are classified as biological, physical and chemical treatments. These methods can be used alone or in combination and can be divided into two main groups: *in-situ*, in which the contaminated medium is treated in place and *ex-situ*, where the contaminated medium is removed from its site of origin and then treated with a

suitable remediation method (Khan et al. 2004). Selection of an appropriate remediation technique depends upon: 1) characteristics of the site and the environmental media; 2) characteristics of the contaminant and extent of contamination at the site; 3) regulatory requirements; 4) cost of operation and 5) time required for remediation (Khan et al. 2004). These *in-situ* and *ex-situ* methods are further classified according to the environmental media as described below.

Soil remediation techniques

Soil washing is a traditional *ex-situ* remediation technique which includes washing of soil using water combined with solvents which solubilize specific contaminants. Soil washing helps to separate fine soil (clay and silt) to which hydrocarbon contaminants tend to bind from coarse soil (sand and gravel) and thus helps to reduce the volume of contaminant (Chu and Chan 2003).

Soil vapor extraction (SVE) is an accepted and cost efficient *in-situ* technology for remediation of soil contaminated with volatile organic compounds (VOCs). SVE involves application of a vacuum through wells installed in the soil near the source of the contamination to extract the volatile contaminants. Extracted vapors withdrawn through an extraction well are treated by carbon adsorption before releasing into the air (Khan et al. 2004).

Land-farming is also an *ex-situ* soil remediation technique where excavated contaminated soil is spread into thin layers on an impermeable surface to increase the surface area and enhance microbial degradation of a contaminant through aeration and addition of nutrients. Land-farming has been successfully used worldwide to remediate petroleum hydrocarbon contaminations (US EPA 1998b).

Soil flushing is an *in-situ* remediation technique in which extraction fluid is passed through contaminated soil using injection or infiltration processes. Groundwater extraction wells are used to pump groundwater and extraction fluids with adsorbed contaminants to the surface. This is followed by treatment of recovered groundwater and extraction fluids using other remediation technologies such as activated carbon and biodegradation (Khan et al. 2004).

Thermal desorption is an *ex-situ* treatment in which excavated soil is heated to temperatures of 100-600 °C in a chamber in order to vaporize organic contaminants and certain metals such as mercury and thereby separating these contaminants from soil (US EPA 1996f). This technique does not destroy the contaminant but converts it to a more treatable form. The vaporized contaminants are then collected and transported by a gas or vacuum system to an offsite treatment system for remediation by other means such as activated carbon.

Phytoremediation is a very effective *in-situ* technology which uses plants to remediate contaminated soil and groundwater by accumulating and degrading these contaminants. There are five types of phytoremediation techniques: 1) rhizofiltration (contaminants are absorbed by plant roots); 2) phytoextraction (contaminants from the soil are transferred to the plants); 3) phytotransformation (degradation or transformation of contaminants by plant metabolic processes); 4) phytostimulation (activities of plants in the root zone stimulate microbial degradation) and 5) phytostabilization (reduces migration of contaminants through soil). Phytoremediation techniques are cost effective and can be used on a wide range of contaminants with minimal environmental disturbance (GWRTAC 1996d, Conesa et al. 2011).

A bioslurry system is an *ex-situ* biological treatment in which contaminated soil is excavated and placed in reaction vessels. Water and other additives including selected bacteria to enhance biodegradation of contaminants are added to the reactor vessel (Khan et al. 2004).

Aeration is a well-developed *ex-situ* technique, in which the contact area between soil or water and air is increased to promote the volatilization of contaminants into the soil. The contaminated soil is spread thinly and tilled or turned to increase the rate of evaporation and biological degradation (Khan et al. 2004).

Composting is an *ex-situ* bioremediation technique in which organic contaminants (for example, PAHs) are converted into innocuous and stable byproducts by microorganisms under controlled aerobic or anaerobic conditions. Contaminated soil is excavated and mixed with bulking agents and organic amendments such as wood chips and animal and vegetable wastes. Proper selection of amendments ensures adequate porosity and provides nutrients (carbon and nitrogen) to promote the microbial activity. Maximum degradation efficiency can be achieved by regulating factors like oxygenation, irrigation, moisture content and temperature. Thermophilic conditions (54 °C to 65 °C) are necessary for remediation of contaminated soil by composting. Heat produced by the microbial degradation of the organic material in waste helps to maintain thermophilic conditions (Gestel et al. 2003). Composting is accomplished in aerated static piles, reactor vessels and windrows (FRTR 1995).

Groundwater remediation techniques

Many groundwater remediation techniques include installation of treatment wells in the area of contamination which can be used not only for extraction of contaminant

from groundwater, but also injection of treatment aids to promote the degradation of the contaminant. The pump and treat method is a very common technique in which contaminated groundwater is removed from the site of origin for remediation. After treatment groundwater is either returned to the original site or discharged as surface water or municipal sewage (Khan et al. 2004). Efficiency of the pump and treat technique depends on hydraulic conductivity, transmissivity and hydraulic gradient at the site. This technique is ineffective in fractured rock or clay sites and where contaminants adsorb to soil or have low solubility.

A passive/reactive barrier treatment wall is an *in-situ* method in which treatment walls are installed underground. Contaminated water then passes through this wall and the contaminant is trapped or transformed into harmless substances that flow from the wall. The specific matrix chosen for these walls include granular zero valent iron, activated carbon, granular activated carbon and zeolite (Birke et al. 2003).

Use of groundwater circulation wells is a technology that removes contaminants from groundwater without bringing the water to the surface. The circulation pattern is created by drawing water into a well and pumping it through a well and then reintroducing it to the aquifer without the water reaching the surface. The well is double cased with upper and lower screening intervals. Air is injected through the inner casing, thereby decreasing the density of the groundwater and allowing it to rise. In the rising process, the groundwater passes through the lower screening interval and is partially stripped of volatile compounds. The groundwater then moves upward to the outer casing by passing through the upper screening interval. This hydraulic circulation pattern helps to remove volatile organic compounds (VOCs) in the groundwater (US EPA 1998f).

Ultraviolet-oxidation treatment is an *ex-situ* technique which uses ultraviolet (UV) light in the presence of an oxygen based oxidant (e.g. ozone or hydrogen peroxide) to catalyze the formation of a hydroxyl radical. These hydroxyl radicals can oxidize the contaminant into less harmful compounds (Khan et al. 2004).

In situ groundwater bioremediation techniques are effective in degrading or transforming a large number of organic compounds (petroleum hydrocarbons, non-chlorinated solvents, wood treating wastes, and chlorinated aromatic and aliphatic compounds) to less harmful compounds (Litchfield 2005). Degradation or transformation includes natural processes such as dilution, adsorption and biodegradation to limit the spread of contamination and to reduce the concentration of the contaminant at the site. Biodegradation of contaminants by microorganisms is achieved by fermentation, aerobic and anaerobic respiration. During this process contaminants are broken down by microbial enzymatic reactions. In fermentation reactions, organic compounds can act as both electron donors and acceptors, while in aerobic respiration, oxygen is used as an external electron acceptor (Vidali 2001).

Air sparging is an effective, well established *in-situ* treatment commonly used for remediation of VOCs dissolved in groundwater. This technique involves injecting atmospheric air under pressure creating channels through the contaminated plume as it flows upwards through the water saturated zone. Air sparging promotes biodegradation of VOCs by increasing subsurface oxygen concentrations required by degrading bacteria. The injected air volatilizes the contaminants in the flow channels and transports them to the vadose zone (zone from top of the ground surface to the water table, Figure 2.3) where they can be biodegraded or removed by SVE system. Air

sparging is effective for remediation of a broad range of volatile and semi-volatile contaminants including petroleum hydrocarbons, chlorinated solvents, diesel fuel and BTEX (benzene, toluene, ethylbenzene and xylene) (Kao et al. 2008). This method is very effective at sites with relatively permeable, homogenous soil conditions due to the effective contact between injected air and the media, while heterogeneous soil conditions with low permeability layers reduce the efficiency of air sparging techniques. Another limitation of this technique is shallow groundwater because the number of wells and associated cost required for sufficient coverage increases.

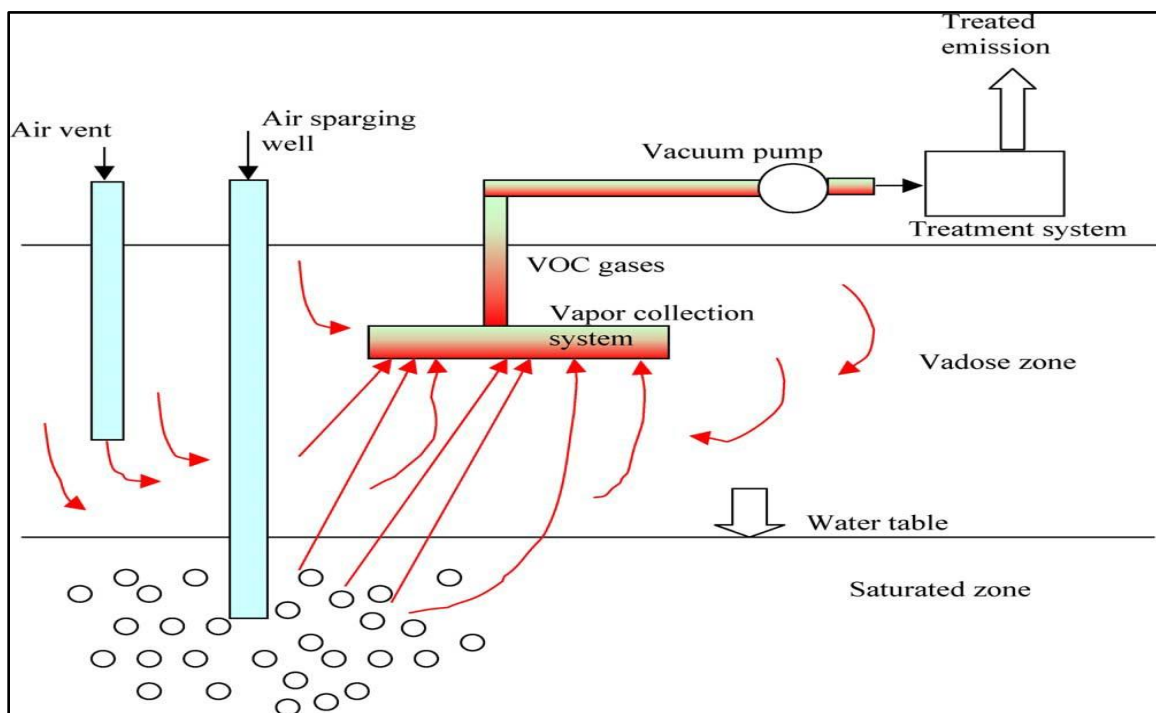


Figure 2.3 Air-sparging with soil vapor extraction process.

Note: Reference: Khan et al. 2004.

Biosparging is a somewhat similar technology to air sparging in which air and nutrients are injected into soil and groundwater to promote the biodegradation of

contaminants by indigenous microorganisms at the site. Biosparging uses low flow rates of injected air to minimize the volatilization of contaminants and enhance biodegradation compared to air sparging. Injected nutrients include nitrogen, phosphates (ortho-P) and potassium which increase the ability of microorganisms to breakdown the groundwater contaminants (Kao et al. 2008). Biosparging has been found to be a good remediation technique for long term control of migrating contaminant plumes that are not easily extracted by physical treatments. Selection of biosparging remediation should be based on soil type, depth of the saturated zone, depth of water table, soil particle size, adsorption potential of contaminant, chemical properties of the contaminant and bacteria present at the contaminated site. In one study, biosparging remediation was evaluated at an abandoned petrochemical manufacturing facility where leakage of a petrochemical pipeline resulted in groundwater contamination with BTEX (benzene, toluene, ethylbenzene and xylene). The biosparging system of six injection wells and 23 monitoring wells was installed within the contaminated plume. The airflow was approximately $0.17 \text{ m}^3/\text{minute}$ for each biosparging well. Natural attenuation of the BTEX concentration occurred at a very slow rate prior to biosparging while BTEX concentrations were significantly decreased after biosparging. Site conditions changed from anaerobic to aerobic as indicated by an increased dissolved oxygen (DO) content, increase in culturable heterotrophic bacteria and decrease in total anaerobic bacteria. Although these contaminants were degraded under both aerobic and anaerobic conditions, the rate of degradation was much higher under aerobic conditions. The significant decrease of more than 70% in BTEX concentration observed within the plume, indicated biosparging to be an efficient remediation technique for this

contaminant in groundwater (Kao et al. 2008). In another study, bioremediation of groundwater contaminated with PCP near a wood treating site using biosparging was evaluated. Seven biosparging wells were installed in a contaminated area and air flow was maintained at 105 standard cubic feet/minute at 15 pounds per square inch (psi). Unidentified PCP degrading bacteria were injected into the sparging wells along with the nutrients to promote biodegradation of PCP. An evaluation period of three and half years indicated a significant and consistent reduction of PCP from approximately 3.3 ppm to 1.650 ppm (up to 50%), indicating efficiency of the biosparging technique to eliminate or minimize offsite migration of PCP in groundwater (Borazjani et al. 2005).

Biodegradation of PCP in groundwater is also influenced by its concentration which impacts its mobility and solubility. At high PCP aqueous concentrations (>20 ppm), biodegradation may not occur because PCP can act as a biocide, while at lower concentrations (0.5- 20 ppm), biodegradation may occur to a greater extent as PCP will be less mobile, more soluble and more bio-available to microorganisms. In areas of very low PCP concentrations (< 0.5 ppm), biodegradation is limited because PCP is likely adsorbed to aquifer materials rather than broken down by microorganisms (Davis et al. 1994).

Table 2.1 Summary of common remediation technologies

| <i>In-situ Technologies</i> | Medium | Application | <i>Ex-situ Technologies</i> | Medium | Application |
|------------------------------------|-------------------|------------------------------------------------------------------------------------------|-----------------------------------------------|---------------|----------------------------------------------------------|
| Soil vapor extraction | Soil | VOCs, SVOCs BTEX Gasoline | Land farming | Soil | Petroleum |
| Soil flushing | Soil | Radionuclides VOCs, SVOCs Fuels Pesticides | Thermal desorption | Soil | Petroleum Mercury VOCs PAHs, PCBs |
| Phytoremediation | Soil | Heavy metals Radionuclides PCBs, PAHs Explosives Cl-solvents Hydrocarbons | Biopiles | Soil | Petroleum VOCs SVOCs Pesticides |
| Bioventing | Soil | Diesel VOCs | Bioslurry systems | Soil | Non-halogenated SVOCs, VOCs Pesticides PCBs |
| Encapsulation | Soil | <i>Not often used</i> | Aeration | Soil | SVOCs Pesticides Fuels |
| Air sparging | Groundwater | VOCs Gasoline Cl-solvents | Pump-and-treat | Groundwater | VOCs, SVOCs Fuels Dissolved metals |
| Passive/reactive treatment walls | Groundwater | VOCs, SVOCs Inorganics | Ultraviolet-oxidation treatment | Groundwater | Petroleum products VOCs, SVOCs |
| Biosparging | Groundwater | Light petroleum compounds | Bioslurping | Groundwater | Floating LNAPL |
| Natural attenuation | Groundwater/ Soil | Fuels Wood treatment wastes Solvents | <i>In-situ or Ex-situ Technologies</i> | Medium | Application |
| <i>Ex-situ Technologies</i> | Medium | Application | Solidification/ stabilization | Soil | Heavy metals Inorganics |
| Soil washing | Soil | SVOCs Heavy metals Petroleum Fuel residues PCBs, PAHs Pesticides | Groundwater circulation wells | Groundwater | VOCs, SVOCs Pesticides Fuels BTEX Inorganics |

Note: Application abbreviations= VOCs – Volatile Organic Compounds, PAHs – Polycyclic Aromatic Hydrocarbons, SVOCs – Semi volatile Organic Compounds, PCBs – Polychlorinated Biphenyls, BTEX – Benzene, Toluene, Ethylbenzene, Xylene Cl-solvents – Chlorinated Solvents. These technologies may be used for treatment of contaminated areas by wood preservatives and other chemicals (Stokes 2011).

Bacterial degradation of PCP

Chemical and physical treatments for remediation of PCP are costly and may produce secondary pollutants. Biological treatments in contrast are comparatively cheaper and environmentally friendly. Prokaryotes in groundwater contribute up to 6% to 40% of the earth's total prokaryotic biomass (Griebler and Leuders 2009). Various types of bacteria exist within the groundwater microbial community such as methane and ammonia oxidizers, lithoautotrophs, denitrifiers, sulfate reducers and methanogens. These bacterial communities are highly adaptive to environmental changes and thus can remediate contaminants like PCP in groundwater. The location of these bacteria within the subsurface environment is also important for remediation of contaminated groundwater, as free living or surface attached bacteria have better opportunity for interaction with contaminants than bacteria that are bound tightly within soil aggregates (Griebler and Leuders 2009). Bacterial remediation may not apply to all remediation treatments, but does play a primary or supporting pathway in many remediation techniques. A description of bacteria present at the site of contamination and understanding the mechanism of degradation by these bacteria would help to increase the efficiency of several remediation techniques.

PCP can act as a source of carbon and energy for some microorganisms which facilitate its degradation. Many bacterial species have been reported to actively degrade PCP and these include various strains of *Burkholderia cepacia*, *Novosphingobium sp.*, *Pseudomonas sp.*, *Sphingobium chlorophenolicum* and *Arthobacter sp.* (He et al. 2008). Recent studies have led to identification of many new strains of bacterial species with ability to degrade PCP in the environment. Karn et al. (2010a) isolated three *Bacillus*

strains identified as *Bacillus megaterium*, *B. pumilus* and *B. thuringensis* from pulp and paper mill secondary sludge using 16S rRNA sequence analysis. These *Bacillus* strains were found to utilize PCP as a source of energy and hence degrade PCP, confirmed by detection of intermediate metabolites of the PCP degradation pathway. These *Bacillus* strains were able to degrade 77% of PCP from the paper mill sludge. A similar study involving bacterial degradation of PCP from the secondary sludge from a pulp and paper mill identified *Pseudomonas stutzeri* strain CL7, which removed approximately 67% of PCP (Karn et al. 2010b). A study conducted by Sharma et al. (2009) focused on physiological characteristics, growth conditions and the PCP degradation pathway by *Acinobacter* sp. ISTPCP-3, isolated from paper mill effluent discharge. In addition, *Burkholderia cepacia* and *Sphingobium chlorophenolicum* ATCC 39723 have also been extensively studied with respect to their PCP degradation mechanisms. In comparison with other PCP degrading bacterial strains reported in previous studies, *Sphingomonas chlorophenolica* has a higher potential for PCP degradation under different growth conditions and hence would be expected to be well suited for bioremediation of PCP.

Bacterial degradation of PCP is affected by pH. It has been observed that *Sphingomonas chlorophenolica* removed 90% of PCP, when the initial pH was 6.9 to 7.6 while it could not remove PCP when the pH value was 9.2 and below 6.0. Thus neutral to slightly alkaline pH may be required for optimum degradation of PCP by this bacterium (Yang et al. 2006). *Sphingomonas chlorophenolica* was previously identified as a strain of *Flavobacterium* sp. Isolates of *Sphingomonas chlorophenolica* ATCC 39723 and other closely related PCP degrading bacterial strains with adoption of 16S rDNA phylogeny for bacterial classification have been divided into multiple genera. *Sphingomonas*

chlorophenolica is now included in the genus *Sphingobium* (Crawford et al. 2007). Members of the genus *Burkholderia* have been used for bioremediation of many recalcitrant xenobiotic compounds such as trichloroethylene (TCE), polychlorinated biphenyl (PCBs), 2, 4, 5-trichlorophenoxyacetate (2, 4, 5-T), PCP and polycyclic aromatic hydrocarbons (O'Sullivan and Mahenthiralingam 2005). Thus the presence of these organisms within contaminated groundwater indicates high potential for microbial degradation of PCP. Other microorganisms with the ability to degrade chlorophenols in addition to *S. chlorophenolica* and *B. cepacia* are beneficial for complete remediation of contaminated sites as these microbes help to degrade smaller chlorinated metabolites produced during degradation of PCP. For example, species like *Pseudomonas fluorescens* and *Collimans sp.* utilize hydrocarbon contaminants as an energy source which helps to degrade the carrier oil (P-9 oil, No. 2 fuel oil, kerosene or mineral spirits) that contains PCP in preservative formulations (Bharathi and Vasudevan 2001).

Chlorophenols are degraded via two main pathways by aerobic bacteria. Lower chlorinated phenols (1- 2 chlorines) are converted into chlorocatechols which are subjected to ring cleavage prior to dechlorination (Figure 2.4) while polychlorinated phenols (3- 5 chlorines) like PCP are converted into chlorohydroquinones and dechlorination occurs prior to the ring cleavage (Karn et al. 2010a, Figure 2.5).

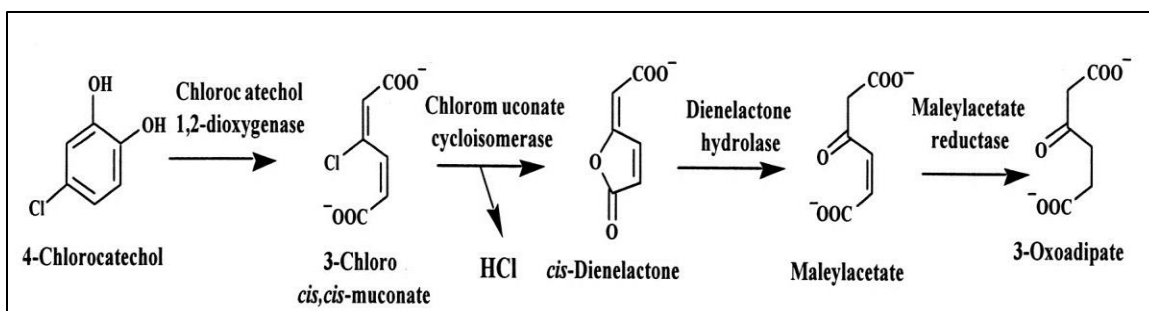


Figure 2.4 Degradation of 4-chlorocatechol by enzymes of the chlorocatechol pathway.

Note: Reference: Nikodem et al. 2003.

Bacterial degradation of PCP follows a stepwise progression of dechlorination of the aromatic ring which is mediated by several oxygenase and reductase enzymes (Figure 2.5). The first step in degradation of PCP is conversion of PCP to tetrachloro-p-hydroquinone (TCHQ) with nicotinamide adenine dinucleotide phosphate (NADPH) as a co-substrate which is catalyzed by the enzyme PCP-4-monooxygenase (Wang et al. 2002, Crawford et al. 2007). On the other hand Dai et al. (2003) suggested that PCP is first converted into tetrachloro-p-benzoquinone (TCBQ) by the enzyme PCP-4-monooxygenase instead of TCHQ. PCP-4-monooxygenase is a flavin monooxygenase that has no substrate specificity. Hydroxylation of phenols by this enzyme is a common step in metabolism of aromatic compounds by soil microorganisms and is the rate limiting step in the biodegradation pathway of PCP (Wang et al. 2002). Bacterial cells that metabolize PCP, accumulate levels of PCP higher than that in the medium, but contain low levels of downstream chlorinated hydroquinone metabolites. This may indicate the slow enzymatic conversion of PCP to TCHQ catalyzed by PCP-4-monooxygenase. This slow and poor enzymatic activity of PCP-4-monooxygenase can be attributed to the reduced catalytic effectiveness because of the broad specificity of the

enzyme. Relatively recent introduction of PCP into the environment may be another reason for the slow enzymatic activity because PCP acts as a new substrate and enzymes have not yet evolved to efficiently degrade PCP (Copley 2000). In the second step of the PCP degradation pathway TCBQ is converted into TCHQ, catalyzed by tetrachlorobenzoquinone reductase. This step is followed by further dechlorination of TCHQ to 2, 3, 6-trichloro-p-hydroquinone (TriCHQ) by TCHQ reductive dehalogenase and then to 2, 6- dichloro-p-hydroquinone (DCHQ) by DiCH dioxygenase. DCHQ acts as a substrate for the ring cleavage enzyme DCHQ dioxygenase and is converted into 2-chloromaleylacetate (2-CMA) which is further converted into maleylacetate (MA) and 3-oxoadipate (3-EXO) by maleylacetate reductase (Chen and Yang 2008).

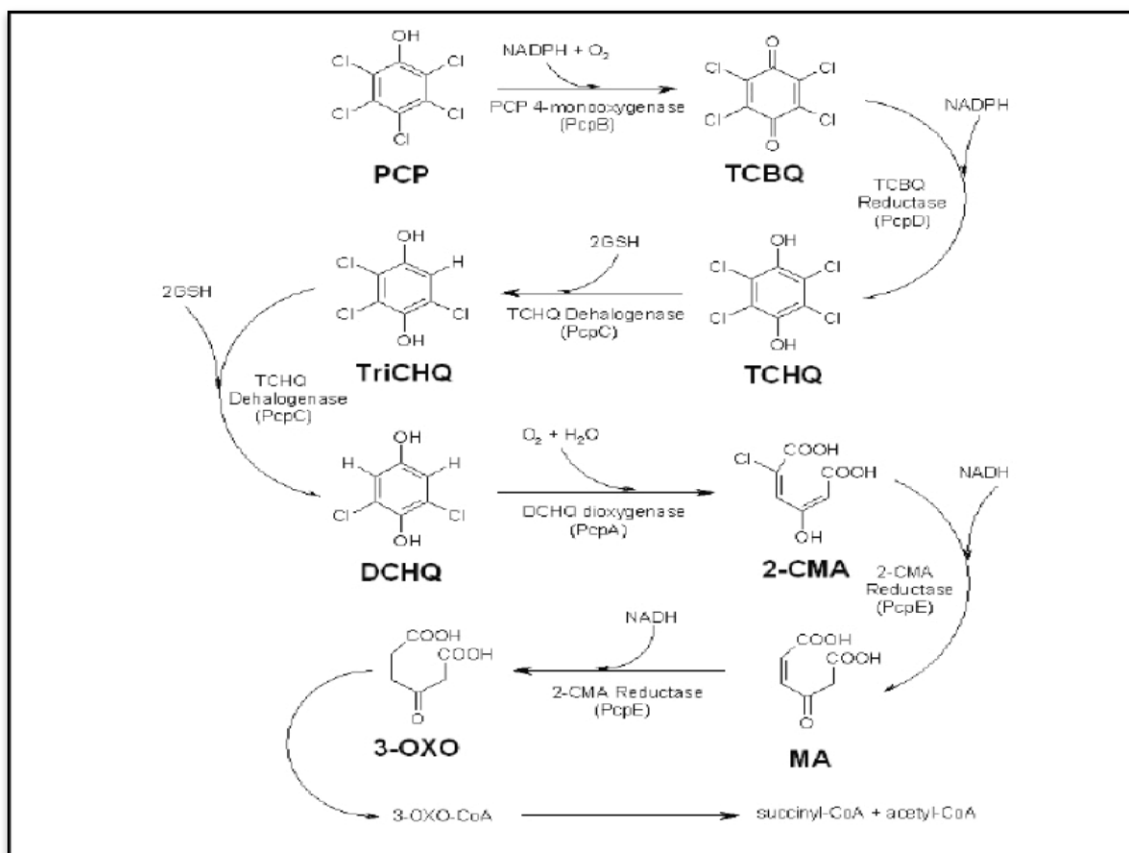


Figure 2.5 The pentachlorophenol (PCP) biodegradation pathway by *S. chlorophenolicum*.

Note: Pentachlorophenol, tetrachlorobenzoquinone, tetrachlorohydroquinone, trichlorohydroquinone, dichlorohydroquinone, 2- chloromaleylacetate, maleylacetate and 3-oxoadipate are represented by PCP, TCBQ, TCHQ, TriCHQ, DCHQ, 2-CMA, MA and 3-OXO, respectively. PcpB, PcpD, PcpC, PcpA and PcpE are genes encoding for enzymes PCP 4- monooxygenase, TCBQ Reductase, TCHQ Dehalogenase, DCHQ dioxygenase, 2- CMA Reductase enzymes (Chen and Yang 2008).

Different genes responsible for the production of enzymes involved in the degradation pathway of PCP have been identified, sequenced and located within the genome of PCP degrading microorganisms. *Sphingobium chlorophenolicum* has been used as a model system to study mechanisms of PCP degradation due to its ability to completely degrade PCP (Cai and Xun 2002). Genes encoding for enzymes for each step

of degradation are shown in Figure 2.5. The enzyme PCP-4-monooxygenase which catalyzes the first step in the degradation pathway is encoded by gene *pcpB*. Various strains of PCP degrading bacteria including many strains of *Sphingobium chlorophenolicum* and *Novosphingobium* sp. MT1 have been found to carry homologues of *pcpB* gene with high levels of similarity between sequences (97-100%) which indicates lateral transfer of the gene among bacteria (Crawford et al. 2007, Tirola et al. 2002). Tetrachlorobenzoquinone reductase which catalyzes the conversion of TCBQ to TCHQ is encoded by *pcpD* gene. TCHQ reductive dehalogenase is encoded by gene *pcpC*, while DCHQ dioxygenase is encoded by *pcpA* gene. The final two steps in the degradation pathway catalyzed by maleylacetate reductase are encoded by *pcpE* gene (Chen et al. 2009). In addition to these genes, certain transcription regulators such as *pcpM* and *pcpR* have been identified. These PCP degrading genes were found to be inducible by the presence of PCP and organized into two clusters, one 24kb fragment containing *pcpE*, *pcpM*, *pcpA*, *pcpC* and one 8 kb fragment containing *pcpB*, *pcpD* and *pcpR*. Strains with mutated forms of these genes and transcription regulators show loss of PCP degradation activity, thus expression of these genes is essential for the degradation of PCP (Cai and Xun 2002). The first step, conversion of PCP to TCBQ regulated by PCP-4-monooxygenase (*pcpB*) is a rate limiting step, thus expression of *pcpB* gene is the most critical step in bacterial degradation of PCP. The presence of *Sphingobium chlorophenolicum* and other PCP degrading bacteria with PCP degrading genes and expression of these genes in PCP contaminated media may indicate possible bioremediation activity for PCP.

Identification of PCP degrading bacteria in groundwater

The composition and the identification of the microbial community present in groundwater is a major challenge. Morphological features and physiological characteristics such as substrate utilization, doubling times and metabolic rate modeling have been used to identify individual bacterial species. However these methods do not provide accurate identification of the groundwater microbial community as many members may have similar functions. The applications of molecular techniques have made identification of different microorganisms more accurate and complete (Amman et al. 1995). Extraction of DNA, amplification of 16S rRNA gene fragments by polymerase chain reaction (PCR, a technique that amplifies specific DNA sequences using oligonucleotide primers), cloning to create a clone library, sequencing of representative library clones and analyses of these sequences using genomic databases are highly accurate methods to identify the members of a microbial community. For identification of bacterial species, the 16S and 23S rRNA fragments are commonly sequenced as these two fragments are highly conserved among bacteria with 1500 and 3000 base pairs, respectively (Macrae 2000). Molecular techniques used for bacterial identification have limitations which affect accuracy. The total number of bacteria in groundwater is generally much lower than in soil (Griebler and Leuders 2009). In addition, microorganisms cultured on selective media represent approximately 0.01 to 1% of total microbial population (Cho et al. 2003). If there are insufficient cells to extract DNA, some species may not be detected using molecular methods. The accuracy of these molecular methods to identify bacteria is highly dependent on the quality of isolated DNA from groundwater bacteria. Selection of primers for the amplification of 16S rRNA

fragment of genomic DNA may not be applicable to every member of a bacterial community (Frey et al. 2006). As part of the identification process, many clones are produced but not all clones are selected for sequencing because this would be time consuming and expensive. Thus some portion of the microbial community may be lost and go unrepresented in the molecular identification process.

Gene expression

The Central Dogma theory of molecular biology helps to explain the mechanism of transfer of genetic information through a biological system. DNA acts a template for its own replication and for transcription to RNA which with subsequent maturation to mRNA serves as a template for translation into enzymes. Expression of genes coding enzymes involved in PCP degradation is needed for enzymatic degradation of PCP. Quantitative real time PCR with an initial reverse transcription reaction is a very sensitive method to determine and quantify gene expression present within a mixed microbial community in the environment. Target gene specificity of this method is determined by use of primers designed specific to the gene of interest. By determining functional genes that encode enzymes in metabolic pathways, a particular microbial function in the environment can be assessed. Thus it helps to link variation in gene expression produced by the PCP degrading bacteria to different environmental conditions (Smith and Osborn 2008). For example, functional genes encoding enzymes for the key reactions in biodegradation pathways of many environmental pollutants have been targeted by RT-PCR analysis. The quantification of these key genes will improve understanding of the potential for the microbial community within the environment for successful in-situ bioremediation and how these indigenous microorganisms respond to bio-stimulation

methods which involve the modification of the environment to stimulate bacteria capable of bioremediation. Lee et al. (2008) quantified expression of reductive dehalogenase (*vcrA*, *bvcA* and *tceA*) genes by using RT-PCR to distinguish the roles of different strains of *Dehalococcoids sp.* during bioremediation of groundwater contaminated with trichloroethane. RT-PCR indicated that *vcrA* and *bvcA* genes were highly expressed in all samples, while the expression of *tceA* genes was at lower levels. This indicated that *Dehalococcoids sp.* carrying *vcrA* and *bvcA* genes may play important roles in trichloroethane in-situ bioremediation of groundwater.

RT-PCR amplification can be conducted by either a one-step or two-step reaction. In one-step RT-PCR, both reverse transcription and quantitative PCR (Q-PCR) is carried out consecutively in a single tube. RNA is first reverse transcribed to cDNA, which then acts as a template for Q-PCR amplification. cDNA has less risk of degradation and can be used to quantify the target gene. However, this method has been found to be less sensitive because two enzymes are in a single reaction (Bustin 2002). In a two-step RT-PCR method, cDNA is generated from isolated RNA by reverse transcription in a separate reaction and subsequently an aliquot of this cDNA is used as template for RT-PCR. In this method, optimization of reverse transcription reaction results in increased cDNA and RT-PCR amplification yield. In addition, cDNA generated in reverse transcription reactions using random primers can be used as a template for several different subsequent RT-PCR amplifications using many gene specific primers. This makes the two-step method more efficient and economic than one-step RT-PCR (Bustin 2002). RT-PCR works as traditional PCR, except the increase in amplicon number is quantified in 'real time' during PCR via detection of a fluorescent signal produced by binding of a

fluorescent reporter to the template. Two commonly used reporters are SYBR green dye and *TaqMan* probe. The fluorescent data from every amplification cycle is collected and the increase in fluorescence is plotted against the cycle number to determine cycle threshold (C_T), which is used to quantify the gene numbers. A housekeeping gene is used to normalize and determine the relative quantification of target gene expression levels in RT-PCR. In case of prokaryotes, 16S rRNA gene is commonly used as a housekeeping gene (Treusch et al. 2005). Even though the use of RT-PCR to quantify the gene expression in an environmental microbial community is common, there are several other factors which can affect the data generated from these reactions. These factors include initial extraction of nucleic acids, preparation, quantification and amplification of template, designing primers specific to target genes, variations in efficiencies of the subsequent Q-PCR, differences in Q-PCR platform, associated software, reagents used and variations due to different researchers and laboratories (Smith and Osborn 2008). Isolation of total RNA from complex environmental samples is typically problematic as RNA is very unstable with a very short half-life. Efficiency of amplification reaction highly depends on preparation of high quality RNA template which is free from any PCR inhibitors (Stults et al. 2001). Dilution of the RNA template to reduce the contamination of inhibitors present may affect the reverse transcription reaction due to low concentration of RNA template. RT-PCR is a very sensitive method, thus it is important that RNA template is free from any contaminated DNA which may contribute to the final amplification signal. The use of a housekeeping gene in relative quantification of gene numbers may not be universally applicable in the case of complex environmental samples because 16S rRNA gene copy and transcriptome numbers are highly variable between

microbial species. The RT-PCR approach is an effective method to determine gene expression and its relation to the potential functional activity of the microbial community in contaminated groundwater, despite some experimental limitations associated with the method.

CHAPTER III

MATERIALS AND METHODS

Site description and collection of PCP contaminated groundwater

PCP contaminated groundwater used in this study was collected at a wood treatment facility in central Mississippi where pentachlorophenol (PCP) had been used to treat utility poles. The collection site has been undergoing air sparging treatment since 2000 after PCP contamination of the groundwater was confirmed. Seven air sparging wells were installed downstream of the contaminant source (an unlined lagoon containing PCP). Eight monitoring wells were installed inside and outside the impact radius of the air sparging wells. Well locations and descriptions are found in Figures 3.1 and Table 3.1.



Figure 3.1 PCP contaminated groundwater sample collection site.

Note: (Top) Site of PCP groundwater contamination indicating groundwater sample collection well MW44 (19AO) (in red circle), monitoring wells (MW, blue circles), air sparging wells (green circles) and direction of groundwater flow (large blue arrows). Well identification code: numbers indicate distance from nearest air sparging well; A, B= above or below air sparging well; I, O= inside or outside air sparging zone of impact. (Bottom) Red arrows indicate distance and direction from air sparging wells to monitoring wells (Stokes 2011).

Table 3.1 Description of monitoring wells

| Well Code | Distance from closest sparging well (m) | Above or below air sparging (A or B) | Inside or outside of treatment zone (I or O) |
|-----------|-----------------------------------------|--------------------------------------|----------------------------------------------|
| 8AI | 8.23 | Above | Inside |
| 19AO | 19.81 | Above | Outside |
| 8BI | 8.53 | Below | Inside |
| 6BI | 6.71 | Below | Inside |
| 7BI | 7.62 | Below | Inside |
| 9BI | 9.14 | Below | Inside |
| 15BO | 15.24 | Below | Outside |
| 17BO | 17.07 | Below | Outside |

Note: Well codes describe three characteristics: 1) distance (m) from nearest air sparging well, 2) Above or Below nearest air sparging well, 3) Inside or Outside air sparging wells zone of impact (approximately 9.14 m radius) (Stokes 2011).

Groundwater sample (19 liters) was collected in a plastic gasoline container (previously rinsed with 70% ethanol and dried) from well number 19AO in December 2011 and stored in a cold-room (4 °C). Groundwater from 19AO was selected for sample collection because it contained the highest concentration of PCP in groundwater collected from the eight monitoring wells (Stokes 2011).

Experimental setup

This study was conducted in two phases: with and without air sparging. The following treatments of PCP contaminated groundwater with three replicates within each treatment were used in this study.

Phase 1: without air sparging (January 2012- July 2012).

1. Treatment 1: Groundwater only.
2. Treatment 2: Groundwater + Miracle Gro™.
3. Treatment 3: Groundwater (autoclaved) + Miracle Gro™ + *Sphingobium chlorophenolicum*.

Each treatment consisted of 900 ml of PCP contaminated groundwater in sterile one liter brown glass bottles. Treatment 1 was groundwater only while treatments 2 and 3 contained groundwater amended with two teaspoons (10 g) of Miracle Gro™ (nitrogen, phosphorous and potassium (15:30:15)) as a nutrient source for bacteria. Groundwater used in treatment 3 was autoclaved twice and then inoculated with one milliliter of pure culture of *S. chlorophenolicum*. All treatment bottles were capped with a sterile sponge and placed in a shaker incubator at 30 °C with shaking at 80 revolutions per minute (RPM) 8 hours per day. There were three sampling times: day 0, 21 and 95. At each sampling time 300 ml of groundwater sample from each treatment bottle was transferred to sterile 500 ml flasks, covered and temporarily stored in a cold-room (4 °C) until analyzed.

Phase 2: with air sparging (July 2012- November 2012)

1. Treatment 1: Groundwater + Miracle Gro™
2. Treatment 2: Groundwater + Miracle Gro™ + *S. chlorophenolicum*
3. Treatment 3: Groundwater + Miracle Gro™ + *B. cepacia*
4. Treatment 4: Groundwater + Miracle Gro™ + Mixed culture of *S. chlorophenolicum* and *B. cepacia*

Each treatment included three sterile brown glass bottles containing 750 ml of groundwater amended with two teaspoons (10 g) of Miracle Gro™. Treatments 2, 3 and 4 were inoculated with 1 ml of *S. chlorophenolicum*, *B. cepacia* and a mixture of these two bacteria (*S. chlorophenolicum* + *B. cepacia*) respectively. Bottles were capped with a plastic cap containing a 3 mm hole which was used for weekly air sparging. All treatment bottles were daily shaken manually for one minute. Treatments 3 and 4 were kept in a locked cooler at room temperature because Bio-safety level 2 (BSL2) regulations are required for *B. cepacia*. There were three sampling times: day 0, 36 and 72. At each sampling time, 250 ml sample from each treatment bottle was transferred to sterile 500 ml flasks and temporarily stored in a cold room (4 °C) for further analyses.

Bacterial strains and growth conditions

Freeze dried bacterial cell pellets of *Sphingobium chlorophenolicum* strain L-1 ATCC® 53874 were obtained from the American Type Culture Collection (ATCC), rehydrated and cultured in the recommended ATCC medium 1687 broth which contained (per liter) 0.65 g of K₂HPO₄, 0.19 g of KH₂PO₄, 0.1 g of MgSO₄·7H₂O, 0.5 g of NaNO₃, 4 g of sodium glutamate (C₅H₈NNaO₄), 2 ml of 0.01 M FeSO₄ and distilled water to make a final volume of 1 liter. Cells were grown in a 30 °C shaker at 200 revolutions per minute (rpm) for 4 days.

Freeze dried bacterial cell pellets of *Burkholderia cepacia* ATCC® 53867 were also obtained from the American Type Culture Collection (ATCC), rehydrated and cultured in the recommended ATCC medium 1694 broth which contained 2.78 g of Na₂HPO₄, 2.78 g of KH₂PO₄, 20 ml of Modified Hunter's Basal salts, 1 g of (NH₄)₂SO₄, 1 g of 2, 4, 5-trichlorophenoxyacetate (C₈H₅Cl₃O₃) and distilled water to make a final

volume of 1 liter. Modified Hunter's Basal salts contained 10 g of nitrilotriacetic acid ($C_6H_9NO_6$), 29.7 g of $MgSO_4 \cdot 7H_2O$, 3.34 g of $CaCl_2 \cdot 2H_2O$, 9.25 g of ammonium molybdate ($(NH_4)_2MoO_4$), 99 mg of $FeSO_4 \cdot 7H_2O$, 50 ml of Metals "44" and distilled water to make a final volume of 1 liter after adjusting the pH to 6.8. Metals "44" used in the above solution contained 0.25 g of ethylenediaminetetraacetic acid (EDTA, $C_{10}H_{16}N_2O_8$), 1.1 g of $ZnSO_4 \cdot 7H_2O$, 0.5 g of $FeSO_4 \cdot 7H_2O$, 0.154 g of $MnSO_4 \cdot 7H_2O$, 0.04 g of $CuSO_4 \cdot 5H_2O$, 0.025 g of $Co(NO_3)_2 \cdot 6H_2O$, 0.018 g of $Na_2B_4O_7 \cdot 10H_2O$ and distilled water to make a 100 ml total volume. *B. cepacia* requires culturing and handling under bio-safety level 2 conditions, thus cells were grown in a locked 30 °C shaker at 150 rpm for 4 days.

Chemical analysis

PCP concentrations in the groundwater samples (200 ml) were determined by EPA standard method 3510C (separatory funnel liquid-liquid extraction) using gas chromatography electron capture detection (GC- ECD) analysis at each sampling time. Each sample was extracted three times with 75 ml of methylene chloride. One ml of 0.5 ppm tribromophenol (TBP) was added as an internal standard to each groundwater sample. After extraction, the combined extract was passed through sodium sulfate into a 500 ml flat bottom flask, reduced to approximately 0.5 ml by condensation using Snyder columns and then transferred to autosampler vials. The volume of each sample was again reduced to approximately 0.1 to 0.2 ml by air, then derivatized by adding 100 μ l of N, O-bis (trimethylsilyl)-trifluoroacetamide + trimethylchlorosilane (BSTFA + TMCS), capping and let sit at room temperature for an hour under a chemical hood. Hexane (900 μ l) was added to the vial to make the final volume of extract up to 1 ml. The processed

samples were analyzed using Agilent 6890 Gas Chromatograph (GC) system. Both internal and external calibration curves generated from 10, 50, 100, 250, 500 and 1000 ppb PCP standards were used to determine the concentration of PCP in groundwater samples.

Bacterial enumeration

In phase 1 of this study, bacterial colony forming units (cfu) were determined by plating 250 µl of 1:1000 dilution of groundwater samples on duplicate plates containing nutrient agar (NA) and nutrient agar amended with 1 ppm of PCP (NA+PCP). In phase 2 bacterial colony forming units were determined by plating 250 µl of undiluted (day 0) and 1:100,000 dilution (day 36 and 72) of groundwater samples on duplicate plates containing NA and NA+PCP. Bacterial colonies on plates were counted after 48 hours incubation at 30 °C, averaged between duplicate plates and corrected for dilution. From this colony count data, the quantity of bacteria in cfu/ml was calculated for both total bacteria and PCP tolerant bacteria.

Identification of bacteria in PCP contaminated groundwater

Bacterial culture and isolation of genomic DNA

One milliliter of groundwater samples at each sampling time was added to 100 ml of sterile nutrient broth media amended with 1 ppm PCP and incubated at room temperature with shaking for 48 hours. The resulting bacterial culture was divided into two 50 ml centrifuge tubes and concentrated to 10 ml by centrifugation at 9000 x g for 10 minutes followed by removing 90 ml of the liquid media. This concentrated bacterial

culture (850 µl) was distributed into twelve 1.5 ml microcentrifuge tubes containing 150 µl of glycerin and stored at -20 °C.

Genomic DNA from the bacterial cultures was isolated using a modified method with the NucleoSpin® Tissue nucleic acid purification kit (Macherey Nagel PA). One milliliter of concentrated bacterial culture was centrifuged at 8000 x g for 10 minutes in a 1.5 ml microcentrifuge tube. Supernatant liquid medium was carefully removed and the cell pellet was resuspended in 180 µl of Buffer T1 by pipetting. Pre-lysis of cells was achieved by addition of 25 µl Proteinase K followed by vortexing and incubation at 56 °C for 1- 3 hours with shaking. Buffer B3 (200 µl) was added and the resulting mixture was vortexed and incubated at 70 °C for 10 minutes to completely lyse the cells. After lysis, DNA was precipitated by adding 210 µl of 96% ethanol and vortexing. For each sample one NucleoSpin® Tissue column was placed into a collection tube. Samples were loaded on these columns and centrifuged for 1 minute at 11,000 x g. Flow-through was discarded and the column was returned to the same collection tube. The column was washed by adding 500 µl of Buffer BW and centrifuging at 11,000 x g for 1 minute. Flow-through was again discarded and the column was again returned to the same collection tube. The column was washed with 600 µl of Buffer B5, centrifuged for 1 minute at 11,000 x g and the flow-through was discarded. The column was placed back into the collection tube and then centrifuged at 11,000 x g for 1 minute to remove any residual ethanol and to dry the column. The column was placed into a new sterile 1.5 ml microcentrifuge tube and 100 µl of Buffer BE (pre-warmed to 70 °C, 5 mM Tris/HCl, pH 8.5) was added to the center of the column. The column was incubated at room temperature for 1 minute and then centrifuged for one minute at 11,000 x g. DNA quantity and quality was determined

using a NanoDrop™ 1000 UV-Vis spectrophotometer (Thermo Scientific, DE). DNA sample (2 µl) was placed on the sample pedestal of the NanoDrop™ spectrophotometer and the absorptions at 230 nm, 260 nm and 280 nm were measured. A_{260:280} purity ratio of 1.8 to 2.0 and A_{260:230} ratio of 2.0 to 2.2 was considered as criteria for good quality DNA. These DNA samples were also analyzed on a 1.5% agarose gel containing 12 µl of GelStar® stain (Lonza Rockland, Inc., Rockland, ME). Five microliters of DNA sample mixed with 2 µl of loading dye was loaded on the gel and electrophoresis was carried out at 90 volts for 2.5 hours. The DNA bands were visualized using Molecular Imager® Gel Doc™ XR+ System with Image Lab™ software (BIO RAD Laboratories, Inc., CA). Size of the extracted genomic DNA was determined by comparison with exACTGene® 1Kb plus DNA ladder (Fisher Scientific, Pittsburgh, PA) on the gel.

DNA amplification

Genes encoding the 16S rRNA were amplified from isolated genomic DNA using bacteria specific primers targeting the 16S region. A 16S forward primer (5'-AGATCGATCCTGGCTCAG) and the universal 16S reverse primer (5'-GGTTACCTTGTTACGACTT) were used in a polymerase chain reaction (PCR) to amplify the approximately 1500 bases of the 16S region. A thermocycler (Eppendorf Master Cycler©) was programmed with the following amplification conditions: initial hot start at 95 °C for 2 minutes, pause, denaturation at 94 °C for 45 seconds, annealing at 57 °C for 1 minute, extension at 72 °C for 2 minutes, repeating for 27 total cycles, final extension of 72 °C for 7 minutes and cooling to 4 °C. The hot start mixture was prepared by adding 10 µl nuclease free water and 3 µl of template DNA into a 0.2 ml PCR tube. The PCR master mixture contained 5 µl PCR buffer (10X, 100 mM Tris-HCl, pH 8.3 at

25°C; 500 mM KCl; 15 mM MgCl₂; 0.01% gelatin), 6 µl MgCl₂ (25 mM), 1 µl deoxynucleotide triphosphates (dNTP) mix (10 mM), 1 µl forward primer (1.6 µM), 1 µl reverse primer (1.6 µM), 1 µl bovine serum albumin (BSA, 10 mg/ml), 0.5 µl *Taq* DNA polymerase (5 U/µl), 31.5 µl nuclease free water (Thermo Fisher Scientific Inc., Pittsburgh, PA) and 3 µl DNA template (300 ng) to make a total volume of 50 µl for each reaction. The hot start mixture was placed in a thermocycler for hot start initialization to decrease the annealing time and prevent non-target amplification. The PCR master mixture of the remaining reaction components (47 µl) was then added to each tube during the pause after the hot start step. A negative control was used which contained all reaction components except the DNA template to monitor the accuracy of amplification reaction. Amplification reactions were completed in approximately 2.5 hours. Quality and quantity of this amplified DNA was determined using a NanoDrop™ 1000 UV-Vis spectrophotometer. For visualization of amplified DNA fragment, 5 µl of sample mixed with 2 µl loading dye was loaded on a 1.5% agarose gel containing 12 µl of GelStar® stain. Five microliters of exACTGene® 1 Kb plus DNA ladder was also loaded on the gel to serve as a size marker to verify the size of the amplified products. Electrophoresis was carried out at 90 volts for 2.5 hours and the bands of amplified 16S region of DNA on the gel were visualized using Molecular Imager® Gel Doc™ XR+ System with Image Lab™ software.

Cloning of DNA 16S fragments

Amplified 16S fragments of DNA were cloned into *E.coli* cells using a TOPO TA Cloning® Kit for Sequencing (Invitrogen, CA). Ligation reactions were performed by adding 2 µl (750 ng) of amplified 16S DNA fragment, 2 µl of nuclease free water, 1 µl of

salt solution (1.2 M NaCl, 0.06 M MgCl₂) and 1 µl (10 ng) of pCR 4-TOPO vector into a 0.2 ml PCR tube. These tubes were incubated at room temperature for 5 minutes and placed on ice. Transformation was accomplished by adding 2 µl of the TOPO ligation reaction to a vial of One Shot chemically competent *E.coli* cells (provided with the kit) while on ice. This mixture of the ligation product and competent cells was gently mixed by tapping the vial carefully. A control sample was used with each set of samples and was prepared by adding 1 µl of pUC19 vector to a separate vial of competent *E.coli* cells and gently mixed. These samples were then incubated on ice for 30 minutes and heat shocked at 42 °C for 60 seconds. After heat shock, these samples were kept on ice for 2 minutes and then 250 µl of room temperature S.O.C. medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose) were added to each vial. These vials were tightly capped and then kept in an incubator at 37 °C with horizontal shaking for 1 hour. After incubation, 50 µl and 100 µl of each transformation reaction was plated on separate pre-warmed Luria broth (LB) agar plates containing 100 µg/ml ampicillin and incubated at 37 °C overnight. Five colonies from each plate were picked and inoculated into a separate tube of 4 ml sterile LB medium containing 100 µg/ml ampicillin. These tubes were incubated overnight at 37 °C with shaking at 100 rpm.

Plasmid extraction and enzyme digestion

Plasmids were extracted from the *E. coli* cultures in LB amended with 100 µg/ml ampicillin using a PureLink™ Quick Plasmid Miniprep kit (Invitrogen, CA). The culture (4 ml) was concentrated to 1 ml by centrifuging at 9,000 x g for 10 minutes and removing the liquid medium (3 ml). The concentrated culture was transferred to a sterile 1.5 ml

microcentrifuge tube and centrifuged at 12,000 x g for 10 minutes to obtain a cell pellet. All supernatant liquid medium was removed and discarded and the cell pellet was completely resuspended in 250 µl Resuspension Buffer (R3, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA) containing RNase A. To the resuspended cells, 250 µl of lysis buffer (L7, 200 mM NaOH, 1% w/v SDS) was added and gently mixed by inverting tubes five times. These tubes were incubated at room temperature for 5 minutes followed by the addition of 350 µl of Precipitation Buffer (N4), mixed by inverting, and then centrifuged at 12,000 x g for 10 minutes. The supernatant from each tube was loaded on a separate spin column placed into a 2 ml wash tube. These spin columns were centrifuged at 12,000 x g for 1 minute and flow-through was discarded. The spin column was placed back into the same wash tube, washed by adding 500 µl Wash Buffer W10 (containing ethanol) and incubated at room temperature for 1 minute, followed by centrifugation at 12,000 x g for 1 minute. Flow-through was discarded and the spin column was placed back into the same wash tube. A second washing of the spin column was carried out by adding 700 µl Wash Buffer W9 (with ethanol) and centrifugation at 12,000 x g for 1 minute. Flow-through was discarded and the spin column was again centrifuged at 12,000 x g for 1 minute to remove any residual Wash Buffer W9. The spin column was placed into a clean 1.5 ml recovery tube and 75 µl preheated (70 °C) T.E. buffer (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA) was added to the center of each spin column. After incubation at room temperature for 1 minute, the spin column was centrifuged at 12,000 x g for 2 minutes to recover the purified plasmid DNA. Quality and quantity of the extracted plasmids was checked using NanoDrop™ 1000 UV-Vis spectrophotometer as previously described.

Restriction enzyme digestion of these plasmids was carried out using *EcoRI* restriction enzyme for 1 hour at 37 °C to check the presence of the correct insert. The digestion mixture contained 2 µl 10X buffer H (500 mM Tris-HCl, pH 7.5; 100 mM MgCl₂; 10 mM Dithiothreitol (DTT); 1000 mM NaCl), 1 µl *EcoRI* (5000 Units), 400 ng of plasmid DNA (approximately 2-4 µl) and nuclease free water to make the total volume of 20 µl. Quality of extracted plasmids and restriction enzyme digestion products was checked by gel electrophoresis. Five microliters of sample mixed with 2 µl of loading dye was loaded on a 1.5% agarose gel containing 12 µl of GelStar® stain. An exACTGene® 1 Kb plus DNA ladder was also loaded on each gel and used as a size marker to detect the presence of two digested fragments with total size of approximately 1500 base pairs.

Sequencing of plasmids for bacterial identification

Preparation of plasmids for sequencing was done according to protocol of the GenomeLab™ Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman-Coulter, CA). The sequencing reaction was carried out in a thermocycler (Eppendorf Master Cycler©) using the following program: Initial hot start at 95 °C for 2 minutes, pause, denaturation at 96 °C for 20 seconds, annealing at 50 °C for 20 seconds, extension at 60 °C for 4 minutes, repeating for 30 cycles and followed by holding at 4 °C. Hot start mixture was prepared by adding approximately 200 ng/µl of template plasmid (approximately 1-2 µl) and nuclease free water to make a final volume of 10 µl in a 0.2 ml PCR tube. Separate master mixtures were prepared: a forward master mixture for forward sequencing reaction and a reverse master mixture for reverse sequencing reaction. These master mixtures contained 2 µl of forward (T3) primer (1.6 pMol/µl) or reverse (T7) primer (1.6 pMol/µl) from the TOPO TA Cloning® Kit for Sequencing

(Invitrogen, CA) and 8 µl of DTCS Quick Start Master Mix (provided with kit). The hot start mixtures were placed in a thermocycler for hot start initialization followed by addition of the forward or reverse master mixtures (10 µl) to the respective hot start mixture tubes in the thermocycler during the pause after the hot start initialization step. This sequencing reaction was completed in approximately 3 hours. Stop solution/glycogen mixture was prepared with 2 µl of 3 M sodium acetate (pH 5.2), 2 µl of 100 mM Na₂-EDTA (pH 8.0) and 1 µl of 20 mg/ml glycogen. Five microliters of this stop solution/glycogen mixture was mixed thoroughly with each sequencing reaction in a 1.5 ml microcentrifuge tube. Sixty microliters of cold 95% ethanol from a -20 °C freezer were added to each tube and mixed thoroughly. The mixture was centrifuged at 14,000 x g for 15 minutes at 4 °C to precipitate the plasmid DNA. These tubes were immediately transferred to an ice block. The supernatant was carefully removed and discarded without disturbing the pellet. The pellet was then rinsed twice with 200 µl of 70% v/v cold ethanol and centrifuged at 14,000 x g for 2 minutes at 4 °C. Supernatant was carefully removed and discarded without disturbing the pellet. The pellet was air dried for 10-15 minutes, and resuspended in 40 µl of Sample Loading Solution (SLS, provided with kit) by vortexing or tapping. Each sample was loaded into a separate well of a sequencing plate and covered with a drop of mineral oil. Sequencing plates with samples were loaded into a Beckman-Coulter CEQ 8000 Genomic Sequencer and analyzed using the standard DNA sequencing protocol and the CEQ Genetic Analysis Software. Sequences were then edited by EditSeq™ (DNASTAR, Inc.). ClustalW2 (European Bioinformatics Institute©) was used to align forward and reverse sequences to obtain a consensus sequence for each sample. These sequences were subjected to NCBI's BLAST database

searches and clones with greater than 98% identity match with two or less gaps were accepted and used for identification of bacterial species.

Gene expression analysis

Determination of optimal growth media and PCP concentration

Phase 1

Nutrient Broth (NB) and ATCC 1687 broth media were prepared according to manufacturer's instructions and evaluated for growth of *S. chlorophenolicum* at different PCP concentrations. One hundred milliliters of each media was distributed into 250 ml flasks. Flasks were covered with aluminum foil, autoclaved and cooled to room temperature. After cooling, each flask containing media was amended with concentrations ranging from 0.008, 0.02, 0.1, 0.2, 0.25, 1, 2.5 and 25 ppm of aqueous sodium pentachlorophenate (sodium salt of PCP), respectively. These flasks were inoculated separately with 1 ml of *S. chlorophenolicum* and 1 ml of groundwater sample. All flasks were incubated at 30 °C with shaking at 80 rpm in a shaker incubator for approximately 48 hours. Summary of all media and PCP concentrations used in phase 1 is shown in Table 3.2.

Table 3.2 Summary of different media and PCP concentrations used to culture bacteria in phase 1.

| Sample | Media | PCP (ppm) |
|-------------------------------|---------------------------------|---------------------------------------------|
| <i>S. chlorophenolicum</i> | Nutrient Broth, ATCC 1687 broth | 0.008, 0.02, 0.1, 0.2, 0.25, 1, 2.5, 10, 25 |
| Groundwater treatment samples | Nutrient Broth, ATCC 1687 broth | 0.008, 0.02, 0.1, 0.2, 1, 2.5, 10, 25 |

Phase 2

In the second phase of the study, one milliliter of *B. cepacia* pure culture was inoculated in sterile NB flasks (100 ml) amended with 0.2, 1, 10 and 25 ppm PCP. These flasks were incubated in a locked shaker incubator at 30 °C and 80 rpm for 48 hours.

The effect of PCP addition to the media after bacterial growth on gene expression was also studied. Sterile ATCC 1687 and 1694 broth media were prepared based on manufacturer's instructions and 100 ml of each media was distributed into 250 ml flasks. One milliliter each of *S. chlorophenolicum* and groundwater samples was inoculated in ATCC 1687 broth while one milliliter of *B. cepacia* in both ATCC 1687 and ATCC 1694 broth. PCP (1 and 10 ppm) was added into each flask after 24 hours of bacterial growth. All flasks were incubated in a locked shaker incubator at 30 °C with shaking at 80 rpm for 48 hours. Summary of all media and different concentrations of PCP used in phase 2 is found in Table 3.3.

Table 3.3 Summary of different media and PCP concentrations used to culture bacteria in phase 2.

| Sample | Media | PCP (ppm) |
|-------------------------------|-----------------|----------------|
| <i>S. chlorophenolicum</i> | ATCC 1687 broth | 1, 10 |
| <i>B. cepacia</i> | Nutrient Broth | 0.2, 1, 10, 25 |
| | ATCC 1687 broth | 1, 10 |
| | ATCC 1694 broth | 1, 10 |
| Groundwater treatment samples | ATCC 1687 broth | 1, 10 |

Extraction of RNA

Bacterial cultures grown in different media amended with PCP were obtained as described above and prepared for extraction of RNA using RNAqueous® Kit (Ambion Inc.). Laboratory bench top and pipettors were cleaned by using RNase Away solution (Molecular BioProducts, Inc.) prior to extracting RNA. Bacterial cultures were concentrated to 1/10 of their initial volume by centrifugation at 9,000 x g for 10 minutes. These concentrated cultures were distributed in 1.5 ml microcentrifuge tube with 1 ml of culture in each and centrifuged at 14,000 x g for 10 minutes to obtain a bacterial cell pellet. After removing the liquid media, the cell pellets were stored in a -70 °C freezer and used for RNA extractions. In some samples, cell pellet was too large to be used as starting material for RNA extraction, therefore nuclease free water was used to dilute these samples 1:2 (500 µl of bacterial culture + 500 µl of nuclease free water) or 1:5 (200 µl of bacterial culture + 800 µl of nuclease free water). One milliliter of bacterial culture

was used as a starting material for RNA extraction and centrifuged at 14,000 x g for 10 minutes. Liquid culture media was removed completely to obtain a cell pellet. Lysis of these cells was achieved by adding 300 µl Lysis/Binding solution and vortexing vigorously. In some cases more (approximately 400 µl to 600 µl) Lysis/Binding solution was added to samples and vortexed vigorously to obtain a clear lysate for extraction of RNA. An equal volume of 64% ethanol (approximately 300 µl to 600 µl) was added to this lysate and mixed by gentle vortexing or pipetting. For each sample a filter cartridge was assembled in a collection tube (provided with kit) and lysate/ethanol mixture from previous step was added to the cartridge. This assembly was centrifuged at 14,000 x g for 1 minute to draw the lysate/ethanol mixture through the filter. This step was repeated until all of the mixture passed through the filter. Flow-through was discarded and the filter cartridge was placed into the same collection tube. The filter cartridge was washed by adding 700 µl of Wash Solution # 1 and then centrifuged at 14,000 x g for 1 minute. The flow-through was discarded and filter cartridge was returned to the same collection tube. The filter cartridge was then washed two times by adding 500 µl of Wash Solution # 2/3 and centrifuging at 14,000 x g for 1 minute. Flow-through was discarded and filter cartridge was returned to the same collection tube. After the second washing, the filter cartridge was again centrifuged at 14,000 x g for 30 seconds to remove the last traces of wash solution. The filter cartridge was transferred into a new collection tube (provided with kit). Preheated (approximately 70 °C) elution solution (30 µl) was added to the center of each filter cartridge and centrifuged at 14,000 x g for 1 minute. A second portion (20 µl) of preheated elution solution was added to the center of the filter cartridge and then centrifuged at 14,000 x g for 1 minute. The RNA was immediately kept on ice

to prevent degradation. DNase treatment of the extracted RNA samples was done using the TURBO DNA-free™ Kit (Ambion Inc.) to remove DNA contamination. To each RNA sample 0.1 volumes (approximately 5 µl) of 10X TURBO DNase buffer and 1 µl of TURBO DNase enzyme (2 Units/µl) was added followed by gentle mixing. This mixture was incubated at 37 °C for 25 minutes. Approximately 5 µl of resuspended DNase Inactivation Reagent were then added to the mixture. This mixture was incubated at room temperature for 2 minutes with intermediate gentle mixing 2 to 3 times. After incubation, this mixture was centrifuged at 10,000 x g for 2 minutes. The supernatant containing pure RNA was carefully transferred into a sterile microcentrifuge tube and stored at -70 °C.

Determination of RNA quality and quantity

RNA quality and quantity before and after DNase treatment was determined by using a NanoDrop™ 1000 UV-Vis spectrophotometer as described previously and visualized by gel electrophoresis. RNA (4 µl) was mixed with 1.5 µl loading dye and loaded on 1.5% agarose gel containing 12 µl of GelStar® stain. To determine the size of RNA bands, 5 µL of exACTGene® 1 Kb plus DNA ladder was loaded on the same gel as a size marker. Electrophoresis was carried out at 90 volts for 2.5 hours and a gel picture was captured using Molecular Imager® Gel Doc™ XR+ System with Image Lab™ software.

RNA quality was also checked using the protocol of the Experion™ RNA StdSens Chip Analysis kit (BIO RAD Laboratories, Inc., CA). At the beginning of the procedure, electrodes of the automated electrophoresis station were cleaned by using 800 µl of Experion electrode cleaner solution and DEPC-treated water. RNA gel was filtered by pipetting 600 µl into a spin filter tube (provided with kit) and centrifuged at 1500 x g

for 10 minutes. Gel stain was prepared by adding 1 μ l of RNA stain to 65 μ l of filtered gel in a RNase free 0.2 ml tube and vortexing briefly. This gel stain solution was protected from light at all times. RNA samples and RNA ladder (provided with kit) were prepared by denaturing 3 μ l of each at 70 °C for 2 minutes in a thermocycler (Eppendorf Master Cycler®) and immediately placed on ice. The Experion chip was primed in an Experion priming station after carefully adding 9 μ l of filtered gel stain solution without forming bubbles in the chip's well labeled GS (gel priming well). Priming of the chip was done with a pressure setting of B and a time setting of 1 minute. Gel stain solution (9 μ l) was added into the well labeled GS, while 9 μ l of filtered gel was added into the well labeled G. Loading Buffer (4 μ l) was added to each sample well and RNA ladder well (L) followed by loading 2 μ l of denatured RNA samples and the RNA ladder in their respective wells. A positive control of rat brain tissue RNA (2 μ l) was also loaded in one sample well on the same chip. This chip was vortexed carefully on a vortexer (Experion) for 1 minute and then loaded on the electrophoresis station. RNA samples were analyzed by using the protocol: Prokaryotic Total RNA StdSens. At the completion of the RNA analyses electrodes were cleaned with 800 μ l of DEPC-treated water. Qualitative and quantitative analysis of the data obtained from RNA samples was done using Experion software analysis tool.

cDNA synthesis and amplification

RNA samples were converted into complementary DNA (cDNA) strands and amplified with primers specific to genes coding for PCP degrading enzymes. A library of cDNA was constructed using an iScript™ cDNA Synthesis Kit (BIO RAD Laboratories, Inc., CA) for two step real time PCR. Template RNA of approximately 500 ng (3- 5 μ l)

was used as a starting material and added to a 0.2 ml sterile PCR tube. To this tube 4 μ l of 5X iScript reaction mix (unique mixture of oligo (dT) and random hexamer primers provided with kit) and 1 μ l of iScript reverse transcriptase were added. Nuclease free water (provided with kit) was added to make a final volume of 20 μ l. A negative control was used in which all reagents and template was added except iScript reverse transcriptase. These tubes with all reagents were mixed and transferred to a thermocycler (Eppendorf Master Cycler[©]). Reaction program included 25 °C for 5 minutes, 42 °C for 30 minutes, 85 °C for 5 minutes and cooling at 4 °C. After completion of the reaction program, cDNA was stored in a -20 °C freezer until further analysis. The cDNA sample was amplified by PCR using primers designed specific to genes coding for PCP degrading enzymes. Primers used in this study are described in Table 3.4.

Table 3.4 Gene specific primers (10 mM) used for amplification of cDNA in this study.

| Gene | Forward Primer (5'-3') | Reverse Primer (5'-3') |
|-------------|--------------------------------|--------------------------------|
| 16S | TAGGGTTGGCGATGGCTGA | TTCTTCACACACGCGGCATT |
| <i>pcpB</i> | TGGTGACGTCGGCATTTCGCC | CCCGGCGTCGCCTTCCATT |
| <i>pcpC</i> | CTATGACGACAAGCAGGTGG ACAT | CATCCGCTGATAATAAGCGAG CAG |
| <i>pcpA</i> | CGAACCATATCACCAGTCTG CATC | CATGAAGAAGTCCATGTCCTC CAG |
| <i>pcpE</i> | TCCATATCGGGTTATCTTCGG TCC | ATCGGGATCGTAGACCACGAT CTT |
| <i>pcpD</i> | GGAGACCCGTCATATGACAA ACCCGT | GTCGATCTCGAGGATGTCCAG CACCA |
| TftD | CGGAGGTGGTCGCACGGAAC | CCAGACAACGCGGCCGTCAT |
| 16S HKG3 | GGGCGAACCCTGATCCAGCA | GCACCGGCTTTCACCCCTGA |

Note: References: Cai et al. 2002, Xun and Gisi 2003 and Stokes 2011.

Optimization of annealing temperature was done before amplification of cDNA samples to improve specificity of the amplification reaction. Gradient PCR was used with 12 annealing temperatures: 50 °C, 50.3 °C, 51.4 °C, 53.2 °C, 55.5 °C, 58.1 °C, 60.8 °C, 63.5 °C, 66 °C, 68.1 °C, 69.7 °C and 70.5 °C for amplification of cDNA with the primers in Table 3.4. These amplification products were visualized on a 1.5% agarose gel containing 12 µl of GelStar® as described previously and the annealing temperature setting showing the brightest band of expected size on gel was considered as the optimum

annealing temperature. In this study, an annealing temperature of 63 °C was considered as optimum and used in the amplification reactions.

Amplification of cDNA samples was carried out using a Thermocycler (Eppendorf Master Cycler®) programmed with an initial hot start at 95 °C for 2 minutes, pause, denaturation at 94 °C for 45 seconds, annealing at 63 °C for 1 minute, extension at 72 °C for 2 minutes, repeating for 40 total cycles and final extension at 72 °C for 7 minutes followed by cooling at 4 °C. The master mixture for the PCR contained 5 µl PCR buffer (10X, 100 mM Tris-HCl, pH 8.3 at 25°C; 500 mM KCl; 15 mM MgCl₂; 0.01% gelatin), 6 µl MgCl₂ (25 mM), 1 µl deoxynucleotide triphosphates (dNTP) mix (10 mM), 1 µl forward primer (1.6 µM), 1 µl reverse primer (1.6 µM), 1 µl bovine serum albumin (BSA, 10 mg/ml), 0.5 µl *Taq* DNA polymerase (5 U/µl) and 31.5 µl nuclease free water. Hot start mixture consisted of 2 µl of cDNA template added to 11 µl of nuclease free water in a 0.2 ml PCR tube. This hot start mixture (13 µl) was placed first in a thermocycler followed by the addition of the master mixture (47 µl) to the respective tubes during the pause step. These amplified cDNA products were analyzed by gel electrophoresis. Five microliters of sample were mixed with 2 µl of loading dye and loaded on a 1.5% agarose gel containing 12 µl of GelStar® stain. Five microliters of exACTGene® 1 Kb plus DNA ladder were also loaded on a gel as a size marker to check the bands of amplified products for the expected size.

Real Time PCR (RT-PCR)

Gene expression was analyzed quantitatively by using a two-step Real Time PCR (RT-PCR) procedure (BIO RAD Laboratories, Inc., CA). In the first step a cDNA library was constructed from pure RNA samples as described previously and the second step

consisted of RT-PCR amplification of these cDNA samples with gene specific primers listed in Table 3.4. Real Time PCR was carried out by using iQ™ SYBR® Green Supermix (BIO RAD Laboratories, Inc., CA) and the protocol provided by the manufacturer. A master mixture was prepared containing 12.5 µl of iQ SYBR Green Supermix (2X reaction buffer with dNTPs, iTaq DNA polymerase, 6 mM MgCl₂, SYBR® Green I, fluorescein and stabilizers), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM) and 7.5 µl of nuclease free water. This master mixture (22 µl) was added to each respective well on a RT-PCR plate followed by adding 3 µl cDNA template. Each sample had three replicates. The housekeeping gene used in RT-PCR was 16S gene which is specific for bacteria. Thus two master mixtures were prepared for each sample, one containing primers specific for 16S gene and another with primers specific for the target gene. Thus three replicates of each sample with these two master mixtures were added on RT-PCR plate and loaded on iCycleriQ® System (BIO RAD Laboratories, Inc., CA). Real time PCR program used consisted of an initial denaturation at 95 °C for 3 minutes, denaturation at 95 °C for 45 seconds, annealing at 63 °C for 1 minute, extension at 72 °C for 2 minutes, repeated for 40 cycles and final extension at 72 °C for 7 minutes. Data obtained from this RT-PCR was analyzed using iCycleriQ® System software.

Data analyses

PCP concentrations in groundwater samples at all sampling times were determined using a calibration curve generated from 0.01, 0.05, 0.1, 0.25, 0.5 and 1 ppm PCP standards using Microsoft excel© 2010.

Bacterial identification data was exported into a Microsoft excel© 2010 and analyzed to determine biodiversity measures such as species richness (S), Simpson's

diversity index (D), Shannon's diversity index (H') and species evenness (J) (Hill et al 2003). Species richness is the number of different species present in a community. Simpson's index considers species richness (S) and the relative abundance of each species. It is calculated by the equation,

$$D = 1 - \sum_{i=1}^S p_i^2$$

(Eq. # 3.1)

In this equation, p_i indicates the fraction of all organisms which belong to the i^{th} species, S is the species richness. Shannon's diversity index is slightly different from Simpson's index in that Shannon's index takes into account species evenness along with richness. Shannon's index is calculated by the equation,

$$H' = - \sum_{i=1}^S (p_i \ln p_i)$$

(Eq. # 3.2)

The Shannon's index equation takes into consideration S, the total number of species present, and p_i , the proportion of the i^{th} species to the overall community. Both Simpson's and Shannon's indices were calculated in Microsoft excel© 2010. These Diversity measures were then used to calculate species evenness. Species evenness describes the distribution of individuals among species within the total community and is calculated by the following equation (Magurran 2004),

$$J = \frac{H'}{\log S}$$

(Eq. # 3.3)

H' is the Shannon's diversity measure and S is the average species richness.

Gene expressions of the target genes *pcpB* and *TftD* were determined using C_T values obtained from RT-PCR. Livak method ($2^{-\Delta\Delta CT}$) was used for calculations of normalized relative fold gene expression among treatments (Schmittgen and Livak 2008). Treatment 1 (Miracle Gro™) was considered as a control group as it was not inoculated with known PCP degrading bacteria. Thus using Livak method, expression of target genes relative to the internal control gene (16S rRNA housekeeping gene) in the treatments (2, 3 and 4) was calculated as fold change in gene expression compared to that of treatment 1 (control group). Formula used for Livak method ($2^{-\Delta\Delta CT}$) was,

$$2^{-\Delta\Delta CT} = [(C_T \text{ gene of interest} - C_T \text{ internal control}) \text{ treatments 2, 3 and 4} - (C_T \text{ gene of interest} - C_T \text{ internal control}) \text{ treatment 1}]$$

Correlation between average PCP concentration and average PCP tolerant bacterial colony count as well as correlation between average PCP concentration and normalized relative fold gene expression data was determined by Pearson's correlation coefficient function in Microsoft excel© 2010. Formula used for calculation of correlation coefficient was,

$$CORREL(X,Y) = r = \frac{\sum(x - \bar{x})(y - \bar{y})}{\sqrt{\sum(x - \bar{x})^2 \sum(y - \bar{y})^2}}$$

(Eq. # 3.4)

In this formula, \bar{X}_i and \bar{X} indicate PCP concentration and average PCP concentration for each treatment. While, \bar{Y}_i and \bar{Y} indicate PCP tolerant bacterial colony count or normalized relative fold gene expression and average PCP tolerant bacterial colony count or average normalized relative fold gene expression for each treatment respectively.

Statistical analysis of pH, bacterial quantification, PCP concentration and gene expression data was done using PROC ANOVA and Tukey's studentized Range (HSD) test in SAS version 9.3. PROC ANOVA was used to determine the statistical F and P values to study the differences between average pH values, bacterial colony counts, PCP concentrations and gene expression levels of all treatments over time. Tukey's Studentized Range (HSD) test was used to group the significantly different data points into different groups. Graphical representation of the data was done using Microsoft Excel© 2010.

CHAPTER IV

RESULTS AND DISCUSSION

pH of groundwater samples

In phase 1 (without air sparging) the average pH of the groundwater samples in the treatments was within the neutral range (6 to 8) (Figure 4.1). There were however significant differences observed for the average pH values in treatments containing Miracle Gro™ (TRT 2 (un-inoculated) & TRT 3 (inoculated with *S. chlorophenolicum*), but no significant differences over time in TRT 1 without Miracle Gro™. These differences in pH may have been due to the growth and activity of microorganisms in the groundwater (Gerardi 2006, Wang et al. 2012). Most bacterial communities in the environment show optimum growth at neutral pH. Changes in pH affect the growth of microorganisms by disrupting plasma membranes or inhibiting activity of enzymes and membrane transfer proteins (Antoniou et al. 1990, Gerardi 2006). This effect may have been caused by the Miracle Gro™ which provides the necessary nutrients for growth of microorganisms.

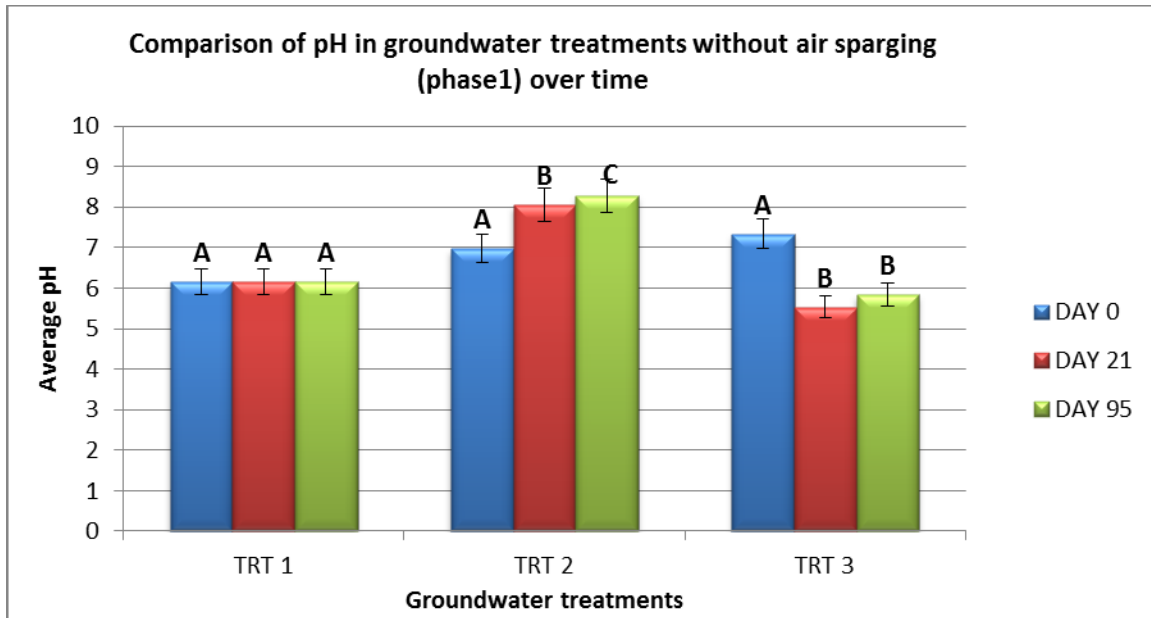


Figure 4.1 Comparison of pH in phase 1 (without air sparging) groundwater treatments on days 0, 21 and 95.

Note: Treatments: TRT 1= without Miracle Gro™, TRT 2= with Miracle Gro™ and TRT 3= with Miracle Gro™ and *S. chlorophenolicum* inoculation. There were three replicates per treatment. Letters A, B and C indicate statistically different pH values of treatments over time. Statistical values: $\alpha = 0.05$ for all treatments. TRT 1: F value= 0.01, F critical= 4.3 and P value= 0.9936; TRT 2: F value= 380, F critical= 4.3 and P value= 0.0001; TRT 3: F value= 15.93, F critical= 5.9 and P value= 0.025.

In phase 2 (with air sparging), the pH ranged from acidic (3.8) at the beginning of the study (day 0) to alkaline (8.4) at the end of the study (day 72) and there were significant differences in average pH values of individual groundwater treatments over the three sampling times (Figure 4.2). These changes in pH may have affected the growth and activity of the bacteria in the groundwater (Hambrick et al. 1980). Prior to the beginning of the study, the groundwater for phase 2 was stored for more than seven months in a closed container which may have created an anaerobic environment, stimulating anaerobic bacterial growth and activity while inhibiting growth of aerobic bacterial communities. Anaerobic and/or facultative aerobic bacteria may have released

organic acids into the groundwater as a result of their metabolic activities causing the pH to decrease (Lowe et al. 1993, Watt and Brown 1985). The increase in pH of the groundwater samples over time (from day 0 to 72), may have been the effect of air sparging creating an aerobic environment and stimulating growth and activity of aerobic bacterial communities. Thus air sparging may have caused a shift in the bacterial population from anaerobic to aerobic and the increase in pH.

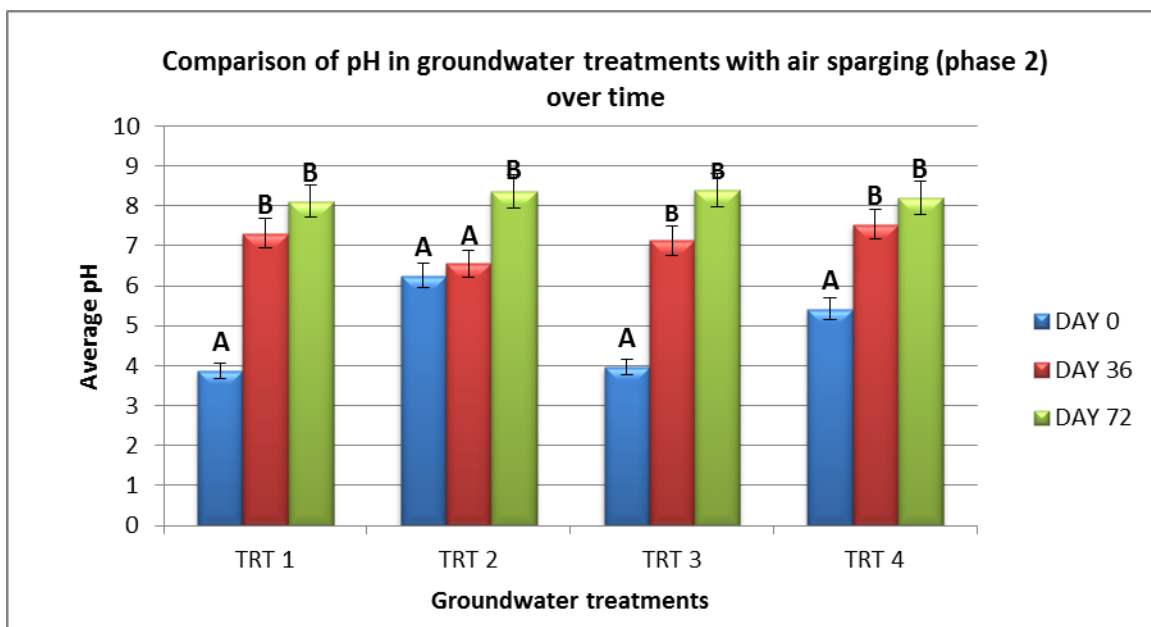


Figure 4.2 Comparison of pH in phase 2 (with air sparging) groundwater treatments on days 0, 36 and 72.

Note: Treatments: TRT 1= with Miracle Gro TM, TRT 2= Miracle Gro TM + *S. chlorophenolicum*, TRT 3= Miracle GroTM + *B. cepacia* and TRT 4= Miracle GroTM + *S. chlorophenolicum* + *B. cepacia*. There were three replicates per treatment. Letters A and B indicate statistically different pH values of treatments over time. Statistical values: $\alpha=0.05$ for all treatments. TRT 1: F value= 88.95, F critical= 4.3 and P value= 0.0001; TRT 2: F value= 27, F critical= 5.9 and P value= 0.011; TRT 3: F value= 8.7, F critical= 4.3 and P value= 0.016; TRT 4= F value= 10.34, F critical= 4.3, P value= 0.011.

Bacterial enumeration and correlation with pH

Total bacteria and PCP tolerant bacteria were determined on nutrient agar (NA) and NA amended with PCP (NA+PCP) plates, respectively. In phase 1 (without air sparging), the average total bacterial count ranged from 0 cfu/ml to 3.6×10^6 cfu/ml (Figure 4.3), while average colony count of PCP tolerant bacteria ranged from 0 cfu/ml to 1.96×10^6 cfu/ml across all treatments (Figure 4.4). Total bacterial colony counts of all treatments over sampling days were higher, as expected than that of PCP tolerant bacteria (Figure 4.5). There were significant decreases between average colony counts of individual treatments over sampling days 0, 21 and 95 (Figures 4.3 and 4.4). Changes in bacterial colony count over time were not uniform. Unlike treatment 1 (without Miracle Gro™) the average colony count of the total bacteria and PCP tolerant bacteria in treatments 2 (Miracle Gro™ + un-inoculated) and 3 (Miracle Gro™ + *S. chlorophenolicum* inoculated) was higher at day 0 as compared to day 21 and 95 indicating significant decrease over time (Figures 4.3 and 4.4).

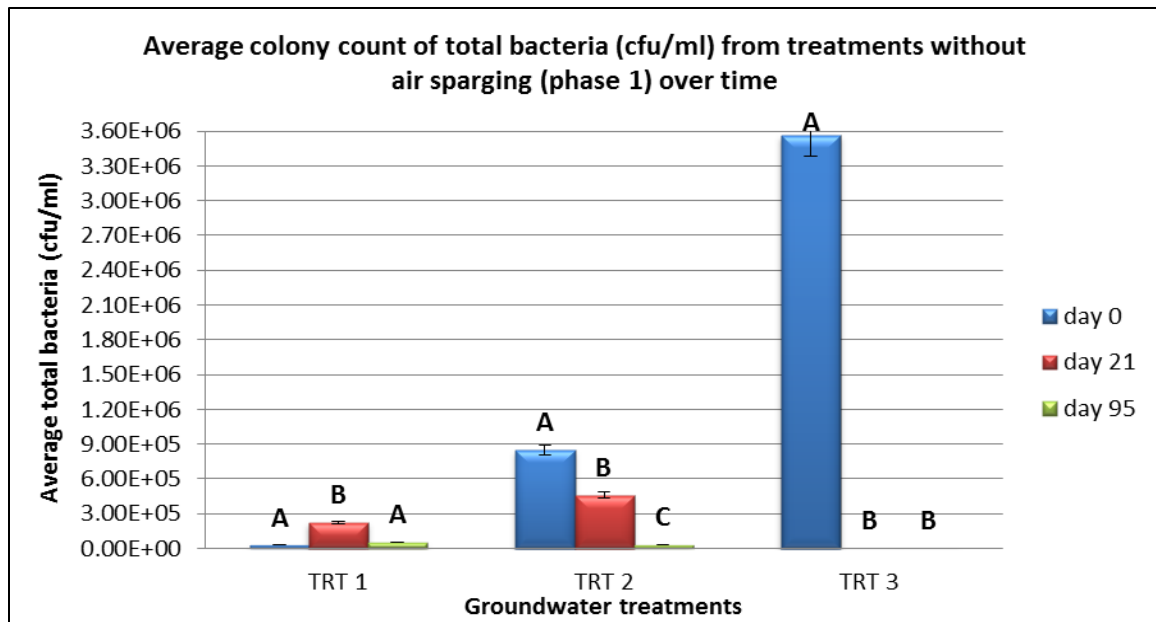


Figure 4.3 Average colony count of total bacteria (cfu/ml) from treatments without air sparging (phase 1).

Note: Treatments: TRT 1= without Miracle Gro™, TRT 2= with Miracle Gro™ and TRT 3= with Miracle Gro™ and *S. chlorophenolicum* inoculation. There were three replicates per treatment. Letters A, B and C indicate statistically different bacterial colony count values of treatments over sampling days 0, 21 and 95. Statistical values: $\alpha=0.05$ for all treatments. TRT 1: F value= 37.54, F critical= 5.9 and P value= 0.007; TRT 2: F value= 50.42, F critical= 4.3 and P value= 0.0002; TRT 3: F value= 121, F critical= 5.9, P value= 0.0014.

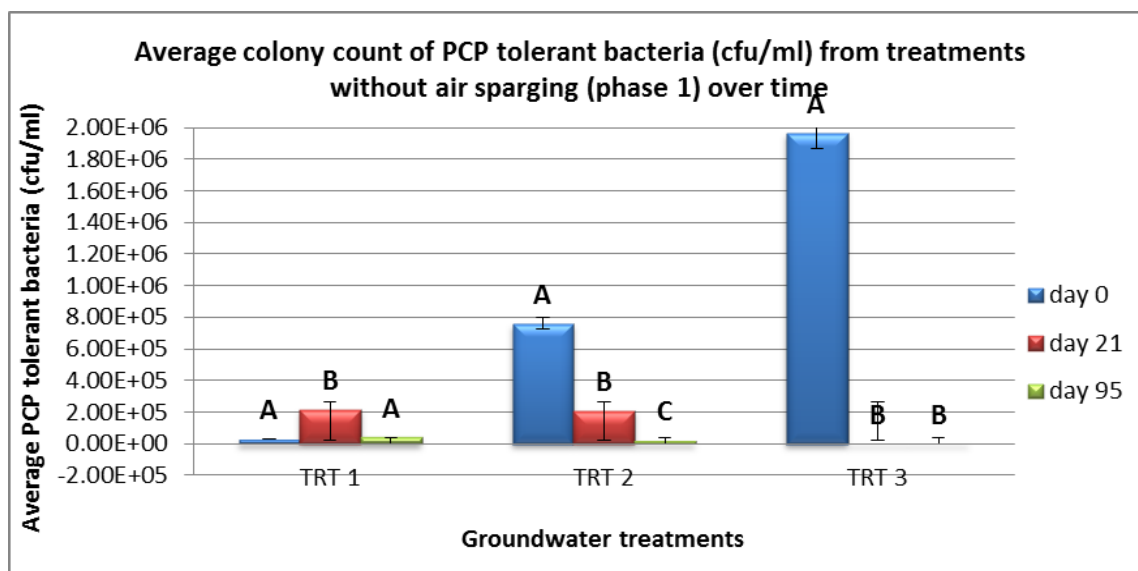


Figure 4.4 Average colony count of PCP tolerant bacteria (cfu/ml) from treatments without air sparging (phase 1).

Note: Treatments: TRT 1= without Miracle Gro™, TRT 2= with Miracle Gro™ and TRT 3= with Miracle Gro™ and *S. chlorophenolicum* inoculation. There were three replicates per treatment. Letters A, B and C indicate statistically different bacterial colony count values of treatments over sampling days 0, 21 and 95. Statistical values: $\alpha = 0.05$ for all treatments. TRT 1: F value= 27.94, F critical= 5.9 and P value= 0.01; TRT 2: F value= 52.77, F critical= 5.9 and P value= 0.004; TRT 3: F value= 394, F critical= 5.9, P value= 0.0002.

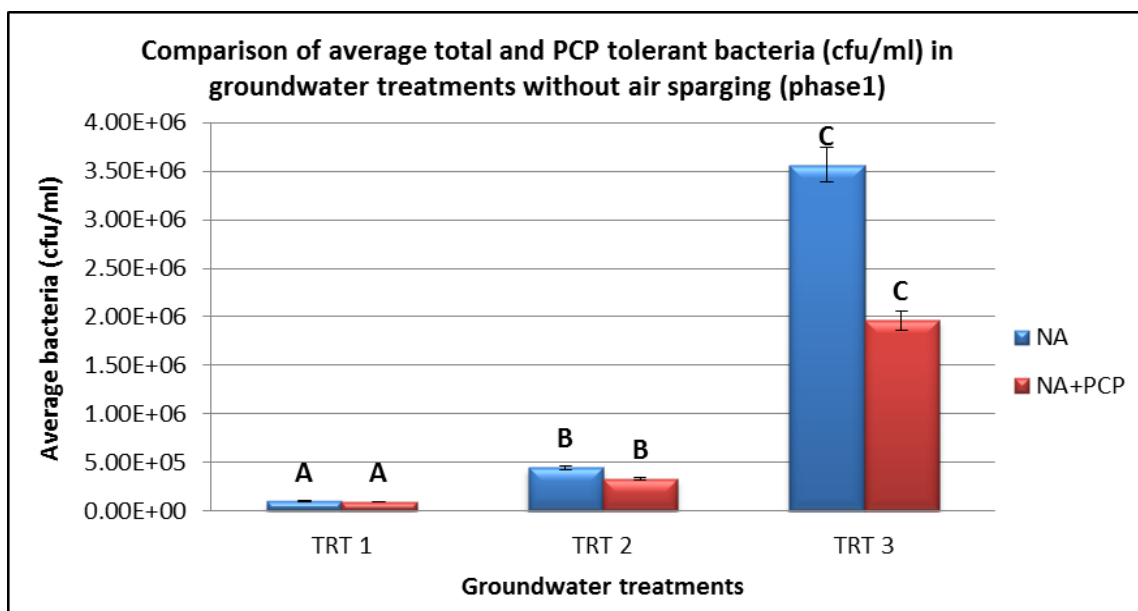


Figure 4.5 Comparison of the average (of sampling days 0, 21 and 95) total and PCP tolerant bacteria (cfu/ml) in groundwater treatments without air sparging (phase1).

Note: Treatments: TRT 1= without Miracle Gro™, TRT 2= with Miracle Gro™ and TRT 3= with Miracle Gro™ and *S. chlorophenolicum* inoculation. There were three replicates per treatment. Letters A, B and C indicate no significant differences between total and PCP tolerant bacterial colony counts. Statistical values: $\alpha = 0.05$ for all treatments. TRT 1: F value= 0.01, F critical= 3.9 and P value= 0.94; TRT 2: F value= 0.13, F critical= 3.9 and P value= 0.73; TRT 3: F value= 0.15, F critical= 3.9, P value= 0.71.

The relationship between the average PCP tolerant bacterial colony count and pH values was determined by CORREL function in Microsoft excel© 2010. A weak negative correlation ($r = -0.3324$) between pH values and the PCP tolerant bacterial colony count was obtained in treatment 1 (Figure 4.6). A strong negative correlation ($r = -0.9970$) obtained in treatment 2 indicated that as pH increases above the neutral pH, the average PCP tolerant bacterial colony count decreased, while a strong positive correlation ($r = 0.9879$) for treatment 3 indicated that as pH decreases below 7.0, the average PCP tolerant bacterial colony count also decreases. Thus increase or decrease in groundwater

sample pH outside the range of neutral pH may have resulted in a decrease in PCP tolerant bacterial colony counts. Treatment 3 replicates which consisted of autoclaved groundwater inoculated with a pure culture *S. chlorophenolicum* and amended with Miracle Gro™, did not show any visible colonies on days 21 and 95 (Figures 4.3 and 4.4). Optimum pH for growth of *S. chlorophenolicum* ranges from neutral to slightly alkaline. In contrast, Treatment 3 had a slightly acidic pH on day 21 and 95 (average of 5.6). Thus acidic pH might be the reason for no visible bacteria on media plates or the *S. chlorophenolicum* could not survive in autoclaved groundwater.

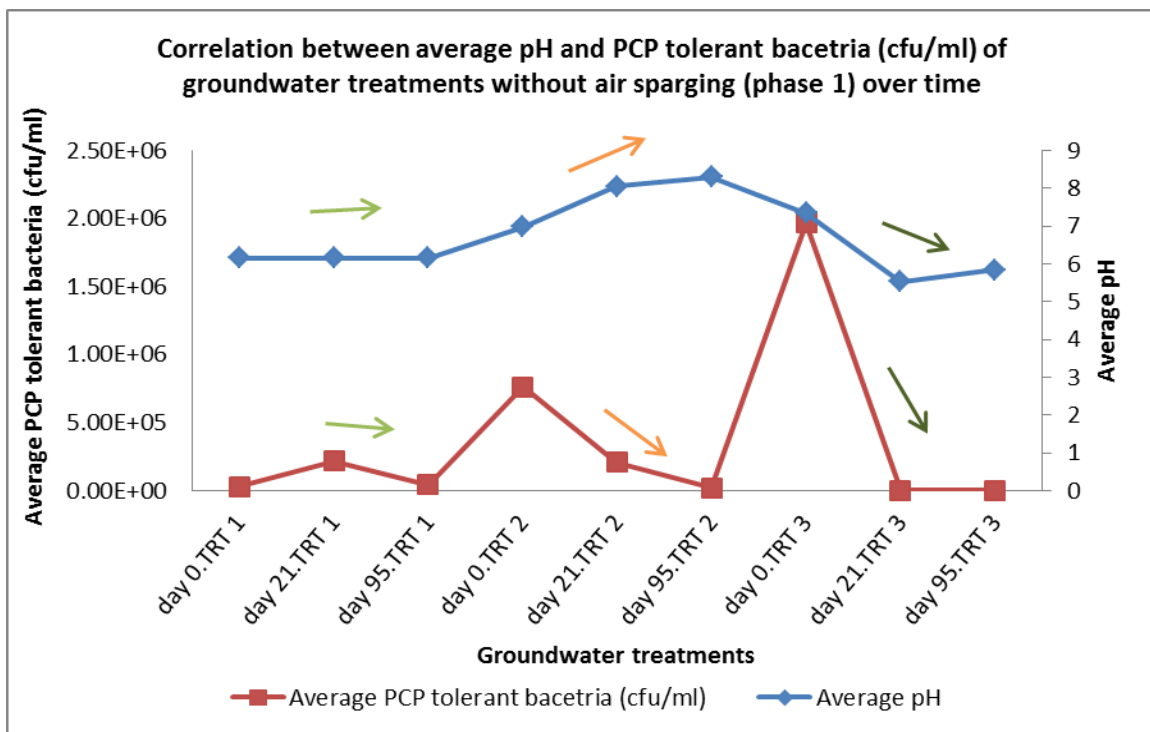


Figure 4.6 Correlation between average pH and PCP tolerant bacteria (cfu/ml) of groundwater treatments without air sparging (phase 1) over time.

Note: Treatments: TRT 1= without Miracle Gro™, TRT 2= with Miracle Gro™ and TRT 3= with Miracle Gro™ and *S. chlorophenolicum* inoculation. There were three replicates per treatment. (Correlation coefficient (r) values: TRT 1= -0.3324, TRT 2= -0.9970, TRT 3= 0.9879).

In phase 2, the average total bacterial colony count ranged from 13 cfu/ml to 1.4×10^8 cfu/ml (Figure 4.7), while the average colony count of PCP tolerant bacteria ranged from 16 cfu/ml to 1.12×10^8 cfu/ml (Figure 4.8). This indicated that nearly all of the bacteria were PCP-tolerant. There were significant differences between average colony counts of individual treatments on sampling day 0 and day 36 for both total bacteria and PCP tolerant bacteria. However no significant differences were observed between average colony counts of individual treatments on sampling day 36 and day 72 for both total bacteria and PCP tolerant bacteria (Figures 4.7 and 4.8). Bacterial colony counts showed an increasing pattern over sampling times (day 0 < day 36 < day 72) (Figure 4.7 and 4.8). Total bacterial counts of all treatments over time were higher than that of PCP tolerant bacteria but they were not significantly different (Figure 4.9).

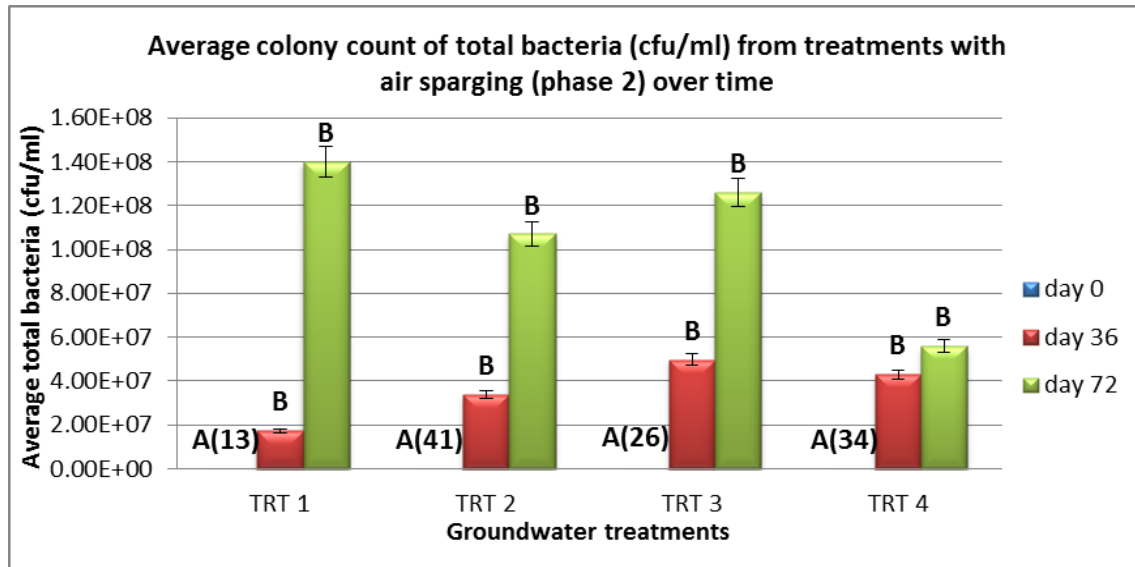


Figure 4.7 Average colony count of total bacteria (cfu/ml) from treatments with air sparging (phase 2).

Note: Treatments: TRT 1= with Miracle Gro™, TRT 2= Miracle Gro™ + *S. chlorophenolicum*, TRT 3= Miracle Gro™ + *B. cepacia* and TRT 4= Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*. There were three replicates per treatment. Letters A and B indicate statistically different colony count values of treatments over time. Statistical values: $\alpha = 0.05$ for all treatments. TRT 1: F value= 13.86, F critical= 6.08 and P value= 0.006; TRT 2: F value= 36, F critical= 6.08 and P value= 0.0001; TRT 4= F value= 19.06, F critical= 6.08, P value= 0.0487 Note: Values in bracket with letter A indicate average cfu/ml of each treatment on day 0.

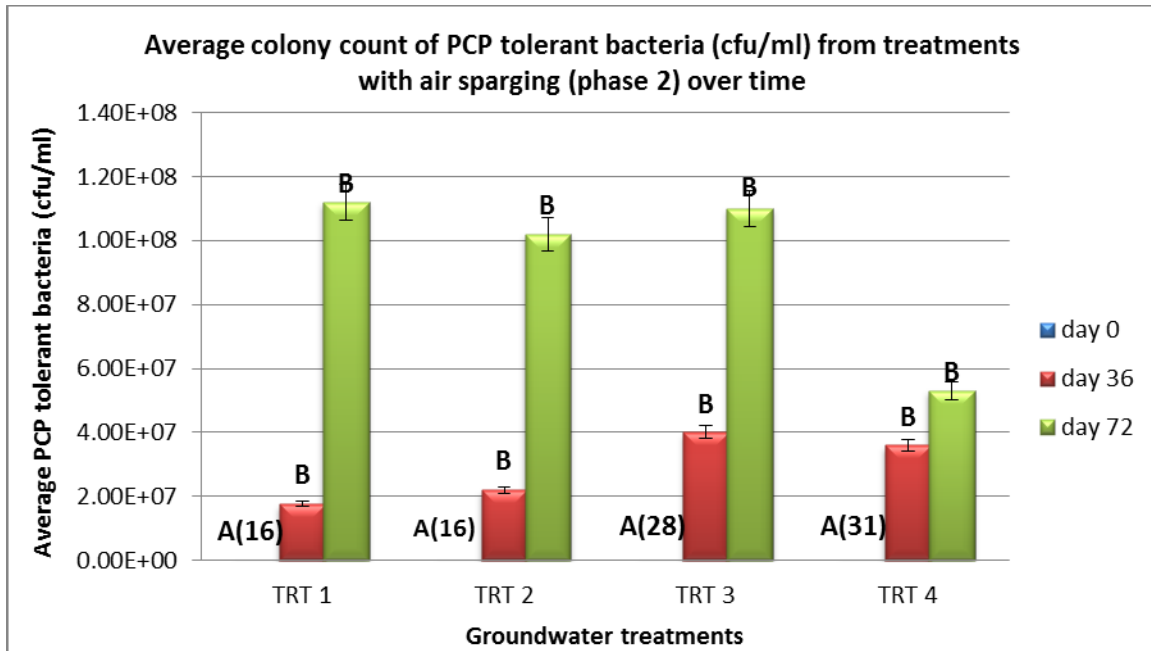


Figure 4.8 Average colony count of PCP tolerant bacteria (cfu/ml) from treatments with air sparging (phase 2).

Note: Treatments: TRT 1= with Miracle Gro™, TRT 2= Miracle Gro™ + *S. chlorophenolicum*, TRT 3= Miracle Gro™ + *B. cepacia* and TRT 4= Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*. There were three replicates per treatment. Letters A and B indicate statistically different colony count values of treatments over time. Statistical values: $\alpha = 0.05$ for all treatments. TRT 1: F value= 12.90, F critical= 5.9 and P value= 0.03; TRT 2: F value= 49, F critical= 6.08 and P value= 0.0198; TRT 4= F value= 45.76, F critical= 6.08, P value= 0.0212. Note: Values in bracket with letter A indicates average cfu/ml of each treatment on day 0.

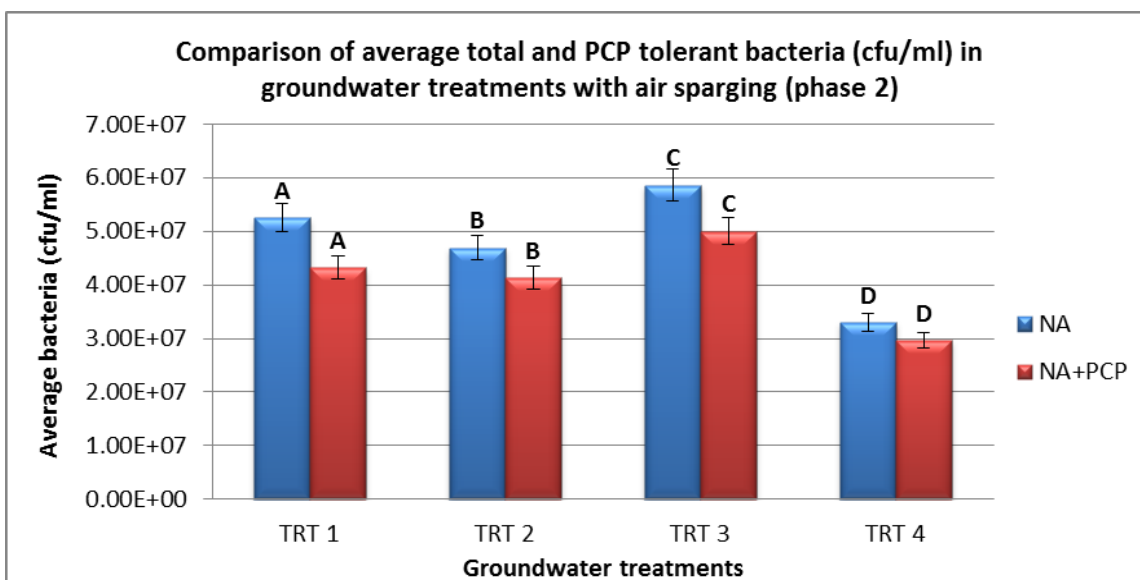


Figure 4.9 Comparison of the average (of sampling days 0, 36 and 72) total and PCP tolerant bacteria (cfu/ml) in groundwater treatments with air sparging (phase2).

Note: Treatments: TRT 1= with Miracle Gro TM, TRT 2= Miracle Gro TM + *S. chlorophenolicum*, TRT 3= Miracle GroTM + *B. cepacia* and TRT 4= Miracle GroTM + *S. chlorophenolicum* + *B. cepacia*. There were three replicates per treatment. Letters A, B, C and D indicate no significant differences between total and PCP tolerant bacterial colony counts. Statistical values: $\alpha = 0.05$ for all treatments. TRT 1: F value= 0.03, F critical= 3.9 and P value= 0.80; TRT 2: F value= 0.02, F critical= 3.9 and P value= 0.90; TRT 3: F value= 0.03, F critical= 3.9, P value= 0.86; TRT 4: F value= 0.02, F critical= 3.9, P value= 0.89.

A strong positive correlation ($r = 0.9176$) was observed between pH and average PCP tolerant bacterial colony count of all treatments over all sampling times. This indicates that from day 0 to day 72, as the pH increased from acidic to alkaline, bacterial colony counts also increased (Figure 4.10). There were no significant differences observed between average pH values of individual treatments on sampling day 36 and 72 except in treatment 2 (Figure 4.2). This may have been the reason for no significant differences between the average colony counts of individual treatments on sampling day 36 and day 72 for both total bacteria and PCP tolerant bacteria (Figures 4.2, 4.7 and 4.8).

Air sparging may have stimulated bacterial growth by increasing the oxygen availability to bacterial communities in the groundwater. The decrease in average PCP tolerant bacterial colonies over time in phase 1 in contrast to phase 2 treatments may have been due to lack of air sparging. Thus optimum pH and aeration may result in an increase in PCP tolerant bacteria which could then enhance the degradation of PCP in the groundwater sample (Hambrick et al. 1980, Stokes 2011).

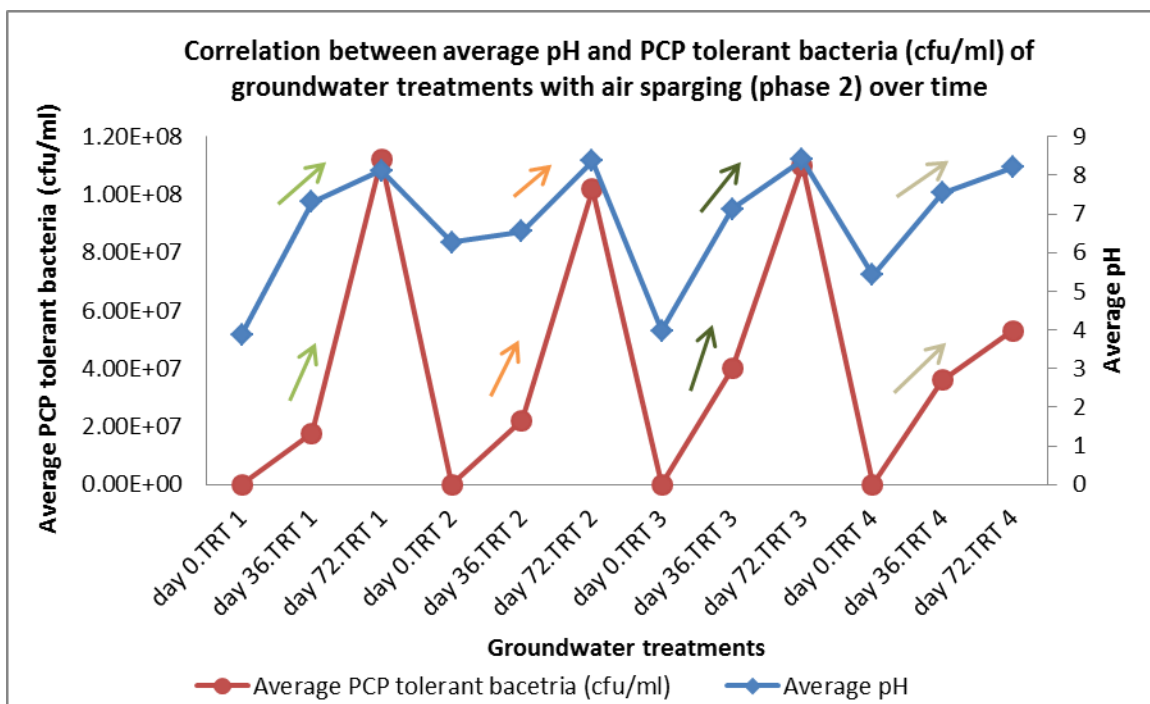


Figure 4.10 Correlation between average pH and PCP tolerant bacteria (cfu/ml) of groundwater treatments with air sparging (phase 2) over time.

Note: Treatments: TRT 1= with Miracle Gro™, TRT 2= Miracle Gro™ + *S. chlorophenolicum*, TRT 3= Miracle Gro™ + *B. cepacia* and TRT 4= Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*. There were three replicates per treatment. Correlation coefficient (r) values: TRT 1= 0.7521, TRT 2= 0.9970, TRT 3= 0.9219 and TRT 4= 0.9959. Average Correlation coefficient (r) value of all treatments over time= 0.9176.

Identification of bacterial community

Identification of bacterial communities in the groundwater samples was performed by cloning and sequencing PCR amplified 16S rRNA gene fragments of extracted DNA. Sequences obtained from CEQ 8000 sequencer were analyzed using NCBI's BLAST database searches and those with greater than 98% identity match and less than 2 gaps were selected as positive matches. Bacterial species and strains identified with their percent identity match obtained in BLAST search are listed in Table 4.1. A few (5.5%) positive matches in BLAST search which were identified as "uncultured" species could not be classified as a particular genus and hence not included in Table 4.1.

Table 4.1 Identification of bacteria in the groundwater based on 98% or greater identity match and less than 2 gaps in the sequence.

| Bacteria | % identity match | gaps |
|------------------------------------------------------|------------------|------|
| <i>Ralstonia eutropha</i> strain HAMBI2380 | 99 | 0 |
| <i>Cupriavidus pauculus</i> strain KPS201 | 100 | 0 |
| <i>Burkholderia</i> sp. LMG 20580 | 100 | 0 |
| <i>Pseudomonas</i> sp. bE19 | 99 | 0 |
| <i>Ralstonia</i> sp. IHB B 2263 | 99 | 0 |
| <i>Bacillus cereus</i> NC7401 | 99 | 0 |
| <i>Burkholderia</i> sp. rif200871 | 99 | 0 |
| <i>Bacillus cereus</i> strain y5 | 99 | 0 |
| <i>Sphingobium chlorophenolicum</i> L-1 chromosome 2 | 100 | 0 |
| <i>Burkholderia</i> sp. SBH-7 | 99 | 0 |
| <i>Bacillus cereus</i> strain GXBC-1 | 99 | 0 |
| <i>Cupriavidus</i> sp. XWS-43 | 100 | 0 |
| <i>Burkholderia</i> sp. TSA69 | 98 | 2 |
| <i>Burkholderia</i> sp. SAP30_2 | 99 | 0 |

Note: 1) % identity match= percent identity match with sequences obtained from NCBI's BLAST database searches. 2) Gaps= number of sequence gaps.

The identification and composition of PCP tolerant bacterial species in the indigenous bacterial community as identified by cloning and DNA sequencing is given in Figure 4.11. The most frequently identified PCP tolerant bacterial species were *Burkholderia* sp. (35%), *Ralstonia eutropha* sp. (20%), *Cupriavidus* sp. (18%), *Bacillus cereus* sp. (18%), *S. chlorophenolicum* (6%) and *Pseudomonas* sp. (3%).

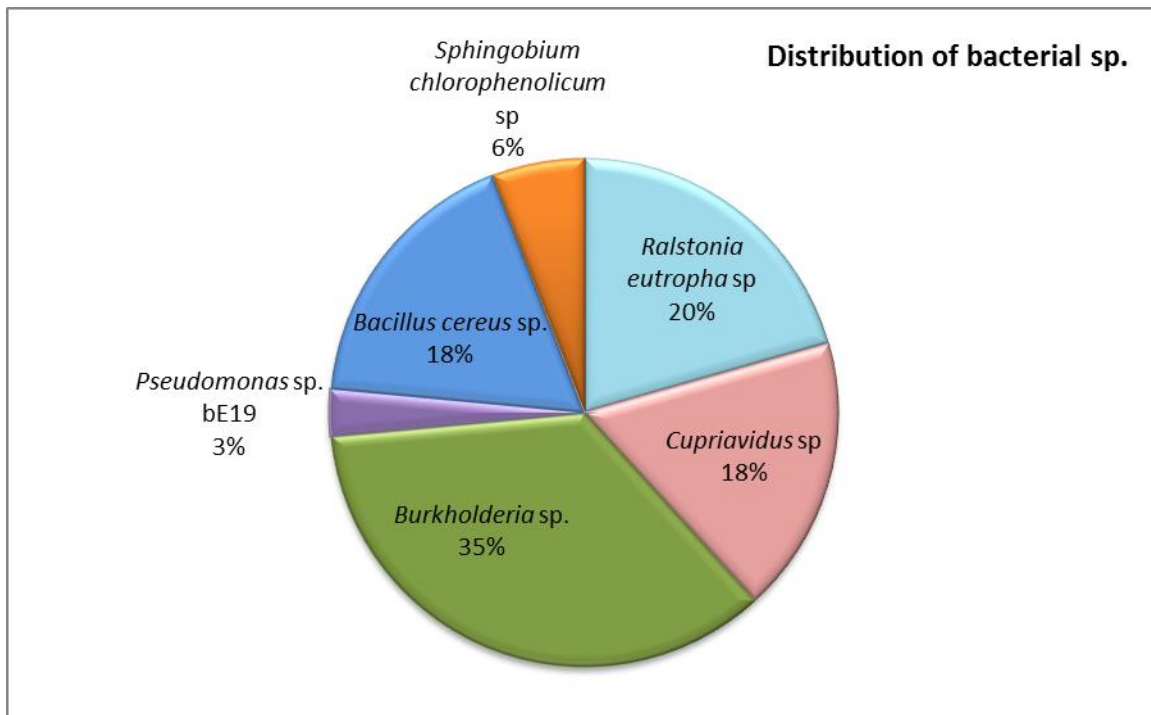


Figure 4.11 Composition of PCP tolerant bacterial species in PCP contaminated groundwater.

Note: *Burkholderia* sp. (35%) was dominant bacteria present in the groundwater community.

Both *R. eutropha* sp. and *Cupriavidus* sp. belong to the order Burkholderiales and hence these are closely related to the *Burkholderia* sp. Both Sphingomonads (*S. chlorophenolicum*) and the Burkholderiales are proteobacteria, but classified into different subclasses. Sphingomonads are alpha proteobacteria while Burkholderiales are

beta proteobacteria. These bacterial species are found in nearly all environments such as polluted waters, soil and groundwater (Jogler et al. 2011). Burkholderiales are facultative aerobic bacteria which means they can survive in anaerobic conditions. This may explain the drop in the pH observed in stored groundwater sample for phase 2 treatments (day 0) (Figure 4.2).

Most bacterial species identified in the study have shown the ability to degrade chlorinated phenols such as PCP and 2, 4, 6-trichlorophenol in the environment (He et al. 2008, Karn et al. 2010 (a, b), Louie et al. 2002, Sanchez and Gonzalez 2007, Xun and Gisi 2003). Among these bacteria, *S. chlorophenolicum* and *Burkholderia* sp. are known PCP degrading bacteria and used as model organisms for investigation of enzymatic degradation of PCP (McAllister et al. 1996, Yang et al. 2006, Chanama M. and Chanama S. 2011). *Bacillus cereus* sp. and *Pseudomonas* sp. however, also have been studied for their ability to degrade PCP (Chandra et al. 2006, Karn et al. 2010a). Thus approximately 62% of the bacterial community in the groundwater is composed of known PCP degrading bacteria which are beneficial for remediation of PCP in the groundwater. (Figure 4.11)

Burkholderia sp. is a dominant and known PCP degrading bacterium present in the groundwater and thus may play a major role in the degradation of PCP in the groundwater sample. Colony counts of PCP tolerant bacteria were higher in Miracle Gro™ amended and air sparged groundwater treatments than in non-amended treatments (Figures 4.5 and 4.9), thus addition of nutrients and air sparging may have been beneficial for growth of PCP degrading bacteria such as *Burkholderia* sp. Similar results were observed for identification of groundwater bacterial communities in the study

conducted by Stokes (2011). *Burkholderia* sp. was a dominant bacteria present in the groundwater and approximately half of the bacterial community was composed of known PCP degrading bacteria.

Biodiversity measures such as species richness (S), species evenness (J'), Simpson's index of diversity (D) and the Shannon-Wiener index (H') were calculated (Hill et al. 2003). Communities with higher Shannon's index values and lower Simpson's index values are more diverse in species distribution. However, species evenness value close to 1 indicates more even distribution of species across the community (Magurran 2004). A Simpson's index (D) value of 0.78 and Shannon's index (H') value of 1.58 indicates a moderate level of biodiversity present in the given groundwater bacterial community. In addition to this, species evenness (J') value of 0.88 also indicates that species distribution in the community was moderately even. This may also indicate that indigenous bacterial community is dominated by one or two bacterial species.

PCP concentration in groundwater treatments

Phase 1 (without air sparging)

In phase 1 the average PCP concentrations in all treatments over time ranged from 0.7 ppm to 1 ppm and are higher than the EPA recommended maximum contaminant load (MCL) of 1 ppb for PCP in the drinking water (Federal Register 54, 1999, Figure 4.12). In treatment 1 of phase 1 (without Miracle Gro™), there was no significant change in the average PCP concentrations over the three sampling times: day 0, 21 and 95 (Figure 4.12). In treatment 2 (with added Miracle Gro™), there were significant decreases among the average PCP concentrations between day 0 and day 21 but not between day 21 and day 95 (Figure 4.12). Thus addition of Miracle Gro™ may have

initially stimulated the PCP degradation by bacteria in the groundwater samples of treatment 2. In treatment 3 (Autoclaved Groundwater + Miracle Gro™ + *S. chlorophenolicum*), there were no significant differences among the average PCP concentrations over time (Figure 4.12). This may have been caused by the absence of *S. chlorophenolicum* as it could not survive at the slightly acidic pH (5.5) of the groundwater samples of treatment 3 on day 21 as compared to day 0 (Figures 4.4, 4.1). PCP concentrations in treatment 3 on the last sampling day (95) were not determined (Figure 4.12) due to lack of sufficient groundwater for analyses. The groundwater had evaporated from the bottles due to their position near the source of heat. While groundwater in other treatment bottles toward the front side of shaker incubator did not evaporate.

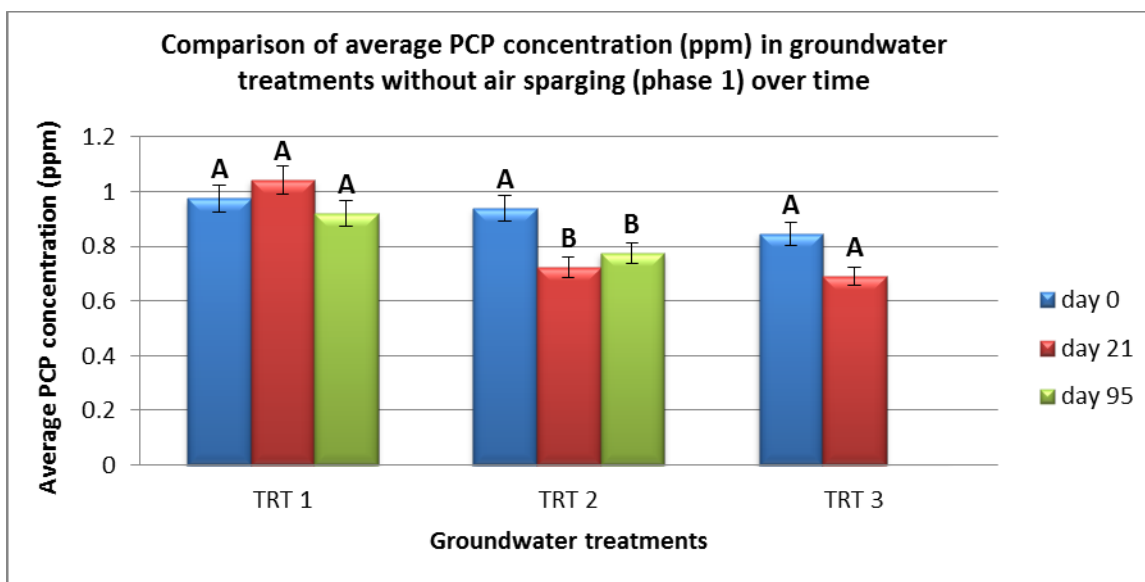


Figure 4.12 Comparison of average PCP concentration (ppm) in groundwater treatments without air sparging (phase 1) over sampling times day 0, 21 and 95.

Note: Treatments: TRT 1= without Miracle Gro™, TRT 2= with Miracle Gro™, TRT 3= autoclaved groundwater + Miracle Gro™ + *S. chlorophenolicum*. There were three replicates per treatment. Letters A and B indicate statistically different average PCP concentration values of treatments over time. Statistical values: $\alpha = 0.05$ for all treatments. TRT 1: F value= 2.19, F critical= 5.9 and P value= 0.25; TRT 2: F value= 10.56, F critical= 5.9 and P value= 0.04; TRT 3= F value= 2.5, F critical= 3.9, P value= 0.18. Note: Data for day 95 samples of treatment 3 were not available.

In phase 1, there were no correlations calculated between average PCP concentration and average PCP tolerant bacteria over time within treatments because the data was not consistent. While, correlation between average PCP concentration and average PCP tolerant bacteria was not determined within treatment 3 over time due to lack of sufficient data (Figures 4.4 and 4.12).

Phase 2 (with air sparging)

In phase 2 the average PCP concentrations in all treatments over time ranged from 0.49 ppm to 0.14 ppm and were higher than the EPA recommended MCL of 1 ppb for

PCP in the drinking water (Federal Register 54, 1999, Figure 4.13). There was no significant decrease in the average PCP concentration of treatment 1 (without Miracle Gro™) over sampling times: day 0, 36 and 72 (Figure 4.13). There were however, significant decreases in the average PCP concentration of treatment 2 (Miracle Gro™ + *S. chlorophenolicum*) (32%) and treatment 3 (Miracle Gro™ + *B. cepacia*) (49%) between days 0 and 72 of 32% and 49% respectively (Figure 4.13). Treatment 3 also showed a significant decrease in the average PCP concentration between days 0 and 36. There was no significant decrease observed in the average PCP concentration of treatment 4 (Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*) over time (Figure 4.13).

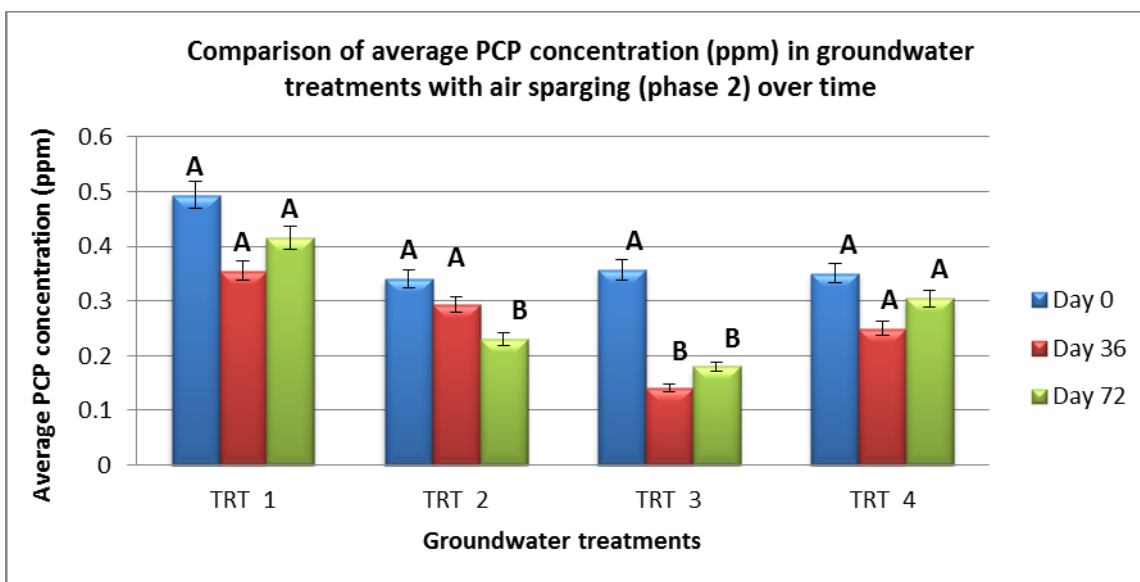


Figure 4.13 Comparison of average PCP concentration (ppm) in groundwater treatments with air sparging (phase 2) over sampling times day 0, 36 and 72.

Note: Treatments: TRT 1= with Miracle Gro™, TRT 2= Miracle Gro™ + *S. chlorophenolicum*, TRT 3= Miracle Gro™ + *B. cepacia* and TRT 4= Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*. There were three replicates per treatment. Letters A and B indicate statistically different average PCP concentration values of treatments over time. Statistical values: $\alpha = 0.05$ for all treatments. TRT 1: F value= 3.9, F critical= 4.3 and P value= 0.07; TRT 2: F value= 19.6, F critical= 4.3 and P value= 0.01; TRT 3: F value= 294, F critical= 4.3, P value= 0.0001, TRT 4: F value= 3.4, F critical= 4.3, P value= 0.1.

Although all phase 2 treatments were air sparged and amended with Miracle Gro™, only treatments 2 and 3 (single inoculum treatments) showed higher PCP tolerant bacteria (Figure 4.8) and significant decreases in PCP concentration over time as compared to treatment 1 (un-inoculated) and treatment 4 (mixed culture inoculum) (Figure 4.13). A strong negative correlation ($r = -0.82$) was observed between the average PCP concentrations and the average PCP tolerant bacteria in treatments 2 and 3 over time indicating that as the PCP tolerant bacteria increased, there was a decrease in PCP concentration (Figure 4.14). Treatments 2 and 3 (single inoculums) were also inoculated

with known PCP degrading bacteria *S. chlorophenolicum* and *B. cepacia* respectively (McAllister et al. 1996, Yang et al. 2006, Chanama and Chanama 2011). Overall this data indicates that addition of a single inoculum of known PCP degrading bacteria in treatment 2 (*S. chlorophenolicum*) and 3 (*B. cepacia*) along with air sparging and Miracle Gro™ increased the degradation of PCP in groundwater samples compared to treatment 1 (un-inoculated) and treatment 4 (mixed culture inoculum).

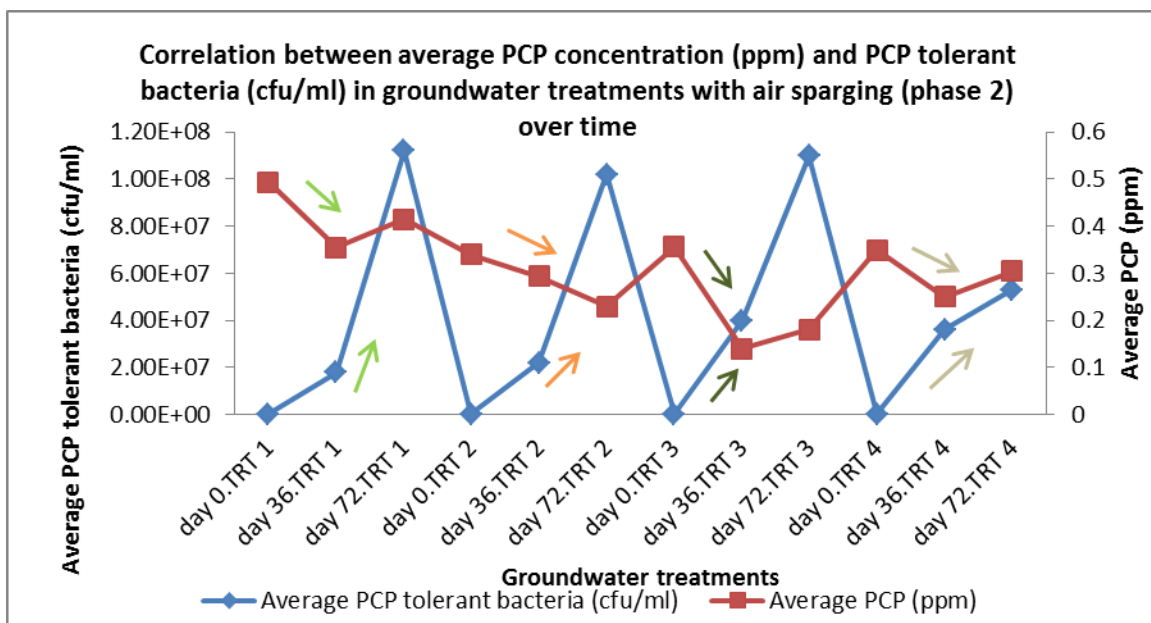


Figure 4.14 Correlation between average PCP concentration (ppm) and PCP tolerant bacteria (cfu/ml) of groundwater treatments with air sparging (phase 2) over sampling times day 0, 36 and 72.

Note: Treatments: TRT 1= with Miracle Gro™, TRT 2= Miracle Gro™ + *S. chlorophenolicum*, TRT 3= Miracle Gro™ + *B. cepacia* and TRT 4= Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*. There were three replicates per treatment. Correlation coefficient (r) values: for TRT 2 and TRT 3 = -0.82, for TRT 1 and TRT 4 = -0.42 and for all treatments = -0.62.

Treatment 4 was inoculated with both *S. chlorophenolicum* and *B. cepacia* (mixed culture inoculum), however there was no significant decrease in average PCP

concentration over time in treatment 4 as seen in treatments 2 and 3 (single inoculum) (Figure 4.13). The average PCP tolerant bacteria in treatment 4 were also less than that of treatments 1, 2 and 3 (Figure 4.9) which may have been a result of an antagonistic effect between *S. chlorophenolicum* and *B. cepacia* resulting in no PCP degradation in treatment 4. Antagonistic effects between microorganisms that degrade environmental contaminants have been studied previously. Slater and Lovatt (1984) reported that while pure individual cultures of *Nocardia* sp. and *Pseudomonas* sp. degraded cyclohexane, a mixed culture of these two bacteria could not, indicating an antagonistic effect between these two bacteria. Wijngaard et al. (1993) studied competitive behavior of *Xanthobacter autotrophicus* GJ10 and *Ancyclobacter aquatics* AD25 and its effect on biodegradation of 1, 2-dichloroethane (DCE). It was observed that due to the antagonistic effect on each other in a mixed culture, no significant degradation of 1, 2-DCE occurred over time.

A weak negative correlation ($r = -0.42$) was observed between average PCP concentration and average PCP tolerant bacteria in treatments 1 and 4 (Figure 4.14). An average negative correlation ($r = -0.62$) between average PCP concentration and average PCP tolerant bacteria in all treatments (1, 2, 3 and 4) over time also indicated that an overall increase in PCP tolerant bacteria correlates with a decrease in PCP concentration (Figure 4.14).

In summary, the significant decrease in average PCP concentrations observed in treatments 2 and 3 was most likely caused by the effect of inoculation of known PCP degrading bacteria, air sparging and addition of Miracle Gro™.

Gene expression of PCP degrading enzymes in groundwater treatments

Phase 1 (without air sparging)

RNA quality

RNA extracted from a pure culture of *S. chlorophenolicum* strain L-1 ATCC® 53874 (positive control) and from groundwater treatment cultures was of good quality and free from DNA contamination as indicated by the presence of distinct 16S rRNA and 23S rRNA bands on the 1.5% agarose gel (Figures 4.15 and 4.16 respectively). RNA from all samples showed purity ratio A260/280 of 1.7 to 2.0 and A260/230 ratio of 1 to 1.8 on the NanoDrop™ spectrophotometer. The quality of RNA was verified using Experion™ RNA StdSens Chip analysis. Similar results showing clear distinct bands for 16S and 23S rRNA regions and 23S/16S ratio of 1.1 to 1.3 indicated the RNA was of good quality (Figure 4.17). RNA was not extracted from treatment 3 samples because of the absence of bacteria on sampling days 21 and 95 most likely caused by slightly acidic pH (5.6) of the groundwater (Figure 4.4).

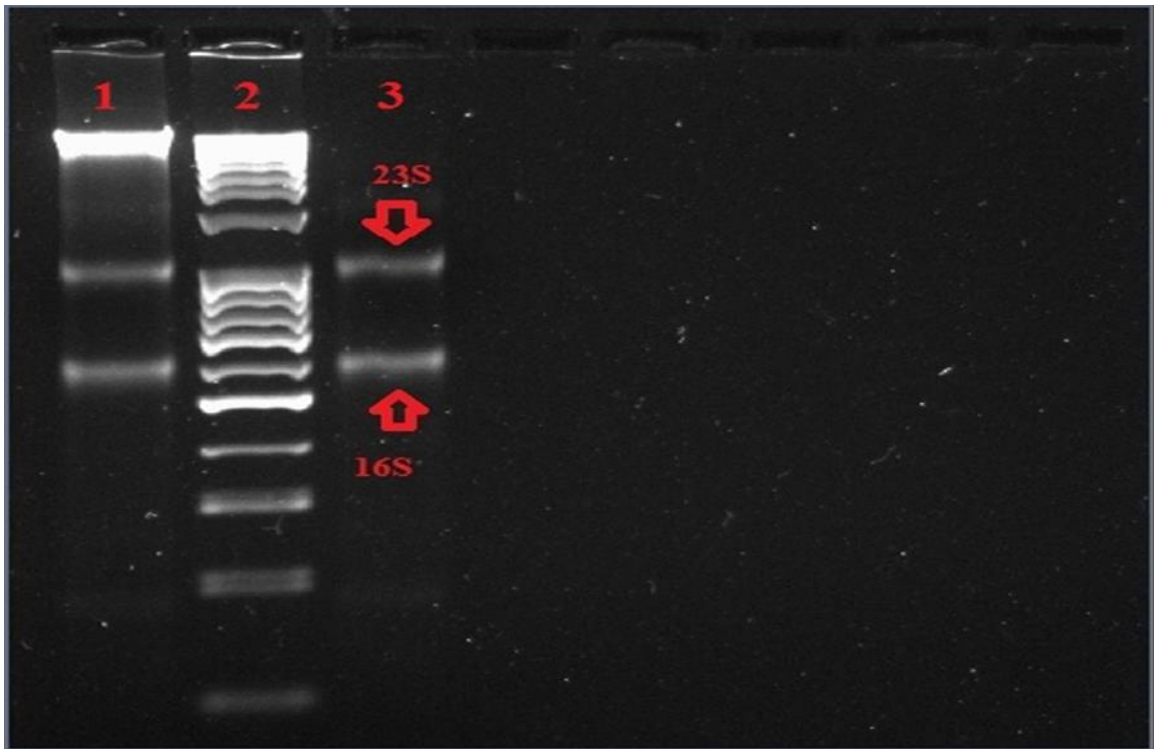


Figure 4.15 RNA extracted from the pure culture of *S. chlorophenolicum* (positive control) in phase 1.

Note: Lane 1 = *S. chlorophenolicum* RNA before DNase treatment; Lane 2 = 1 Kb plus ladder; Lane 3 = *S. chlorophenolicum* RNA after DNase treatment. Red color arrows indicate presence of 16S and 23S rRNA bands.

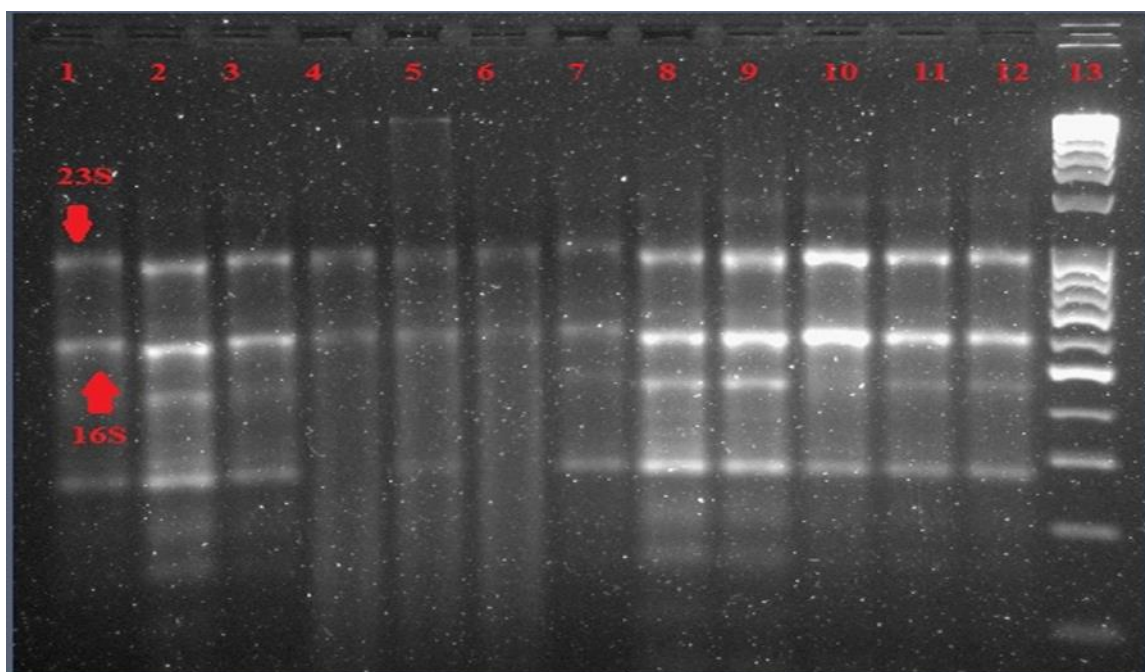


Figure 4.16 RNA extracted from treatments 1 and 2 groundwater cultures of phase 1 (without air sparging) on sampling days 0 and 21.

Note: Lanes 1, 2 and 3 = Day0_TRT 1. rep 1, rep 2 and rep 3; Lanes 4, 5 and 6 = Day0_TRT 2. rep 1, rep 2 and rep 3; Lanes 7, 8 and 9 = Day 21_TRT 1. Rep 1, rep 2 and rep 3; Lanes 10, 11 and 12 = Day 21_TRT 2. Rep 1, rep 2 and rep 3. Lane 13 = 1 Kb plus ladder. Red color arrows indicate 16S and 23S rRNA bands.

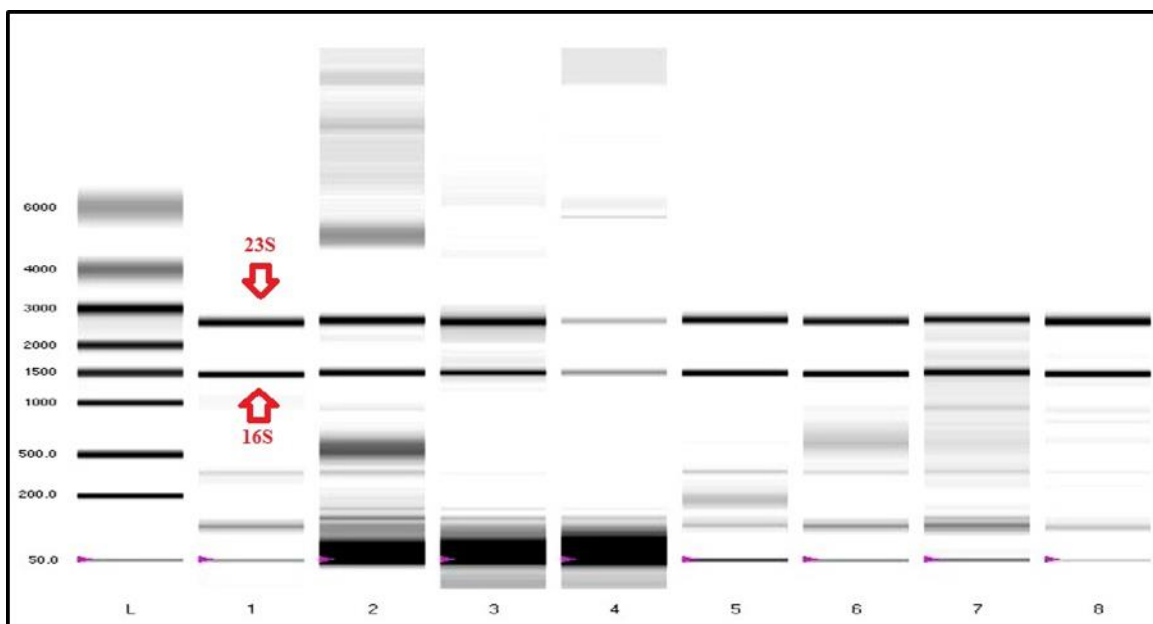


Figure 4.17 Experion RNA StdSens chip analysis of RNA samples from treatments 1 and 2 groundwater cultures of phase 1 (without air sparging) on sampling days 0 and 21.

Note: Lane L= RNA ladder; Lane 1 = day 0_TRT1.rep 2; Lane 2 = day 0_TRT 2.rep 1; Lane 3 = day 0_TRT 2.rep 2; Lane 4 = day 0_TRT 2.rep 3; Lane 5 = day 21_TRT 1.rep 1; Lane 6 = day 21_TRT 1.rep 3; Lane 7 = day 21_TRT 2.rep 1 and Lane 8 = day21_TRT 2.rep 2. Red color arrows indicate presence of 16S and 23S rRNA bands. Note: Light smear in Lanes 2, 3 and 4 indicates slight degradation of respective RNA samples.

Verification of gene specific primers

Amplification of both genomic DNA and cDNA samples from a pure culture of *S. chlorophenolicum* (positive control) using *pcpB* gene (*S. chlorophenolicum* specific gene pentachlorophenol-4-monooxygenase) primers showed bands for the amplified product of the *pcpB* gene (192bp, Figure 4.18). This indicated the presence and expression of *pcpB* gene in the positive control and verified the suitability of the primers for detection of this gene.

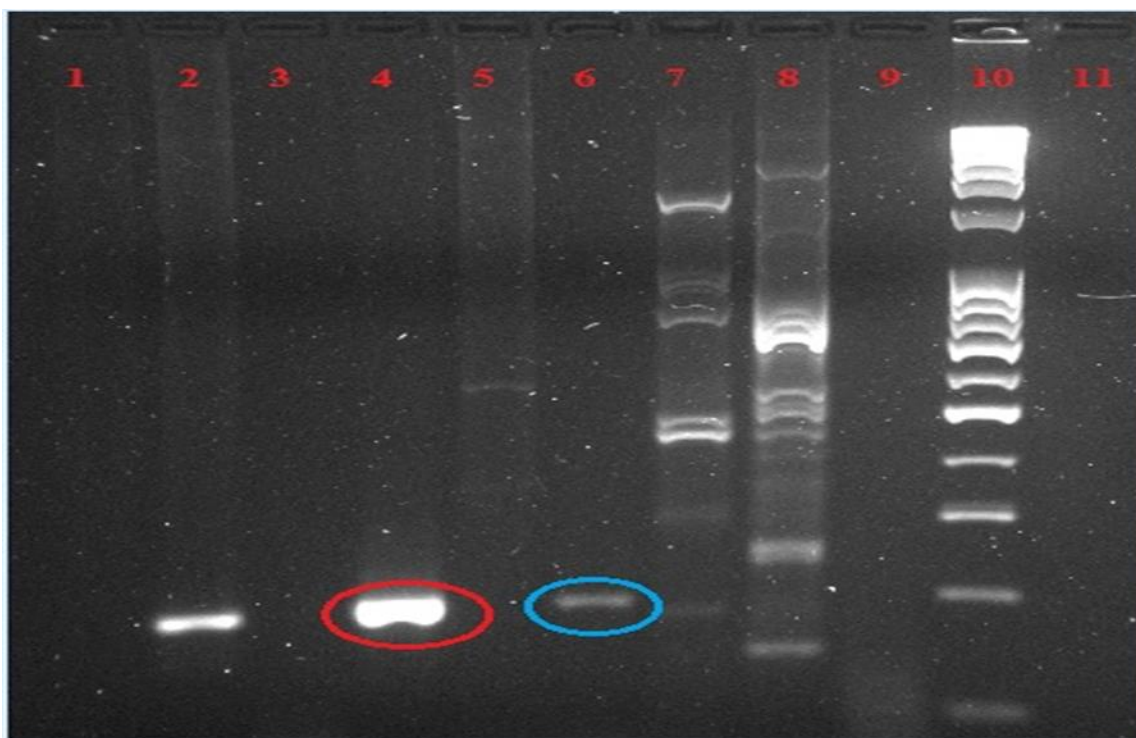


Figure 4.18 Amplification of genomic DNA and cDNA samples of positive control *S. chlorophenolicum* in phase 1 (without air sparging).

Note: Lane 4 = *pcpB* gene in genomic DNA (in red circle); Lane 6 = *pcpB* gene in cDNA (in blue circle); Lane 10 = 1 Kb plus ladder; Lane 11= Negative control for PCR without cDNA template.

DNA samples extracted from groundwater treatments were amplified by PCR using primers specific to genes for 16S rRNA, *pcpB* and TftD (*B. cepacia* specific gene). Bands representing the amplified product of the 16S rRNA gene (173bp, also a housekeeping gene for RT-PCR) indicated the presence of bacterial DNA (Figure 4.19). However bands for the amplified products of the *pcpB* gene (192bp) and TftD gene (185bp) indicated gene specificity of designed primers and the presence of the respective genes in the bacterial DNA (Figure 4.19). The presence of a band for the amplified product of TftD gene (*B. cepacia* specific gene) (Figure 4.19, Lane # 8) indicates the

presence of *B. cepacia* in treatment 2. Although *B. cepacia* was not inoculated in this treatment, it was identified as the dominant bacteria in the groundwater (Figure 4.11).

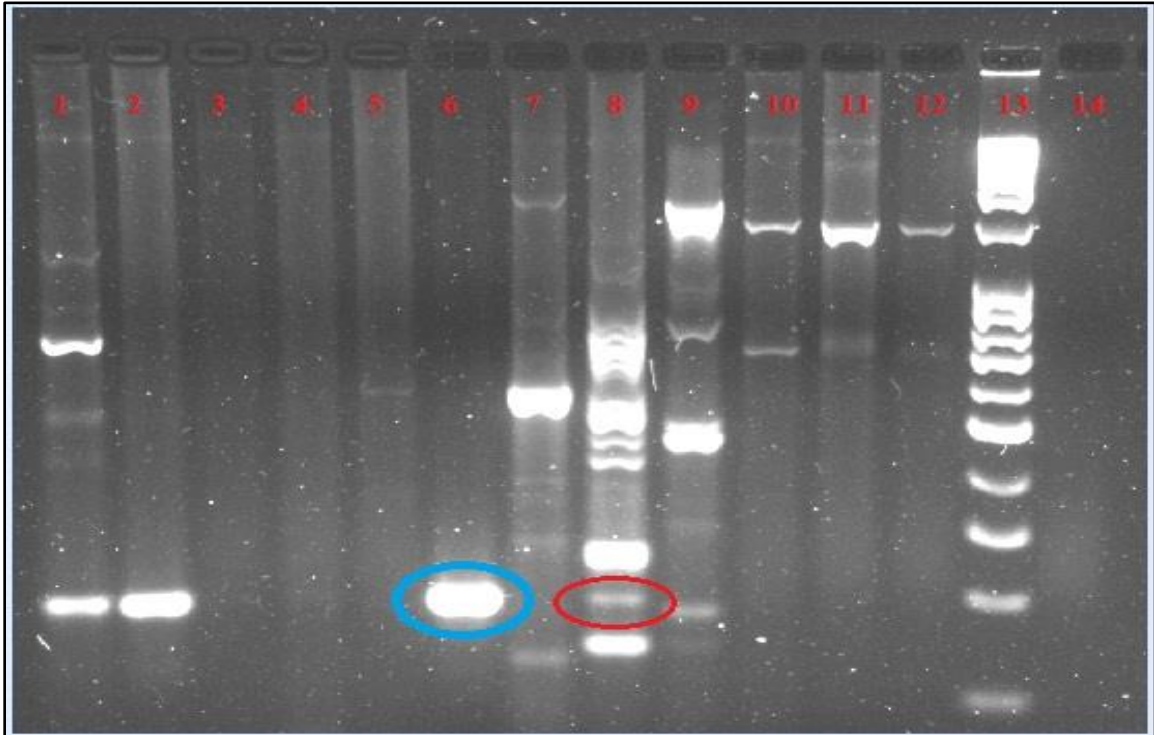


Figure 4.19 Amplification of DNA samples from phase 1 (without air sparging) groundwater treatments by gene specific primers.

Note: Lane 1 = 16S gene in Day 21_TRT 1. rep2; Lane 2 = 16S gene in Day 21_TRT 2. rep 1; Lane 6= *pcpB* gene in *S. chlorophenolicum* (blue circle); Lane 8= TftD gene in Day 21_TRT 2. rep1 (red circle); Lane 13= 1 Kb plus ladder; Lane 14= Negative control for PCR without DNA template.

Quantitation of gene expression using RT-PCR

RT-PCR was conducted on cDNA samples of all treatments on sampling days 0, 21 and 95 with both *pcpB* (*S. chlorophenolicum*) and TftD (*B. cepacia*) gene specific primers. Results indicated that there was no gene expression obtained in RT-PCR as there was no cDNA amplification observed except in case of the housekeeping gene (16S

rRNA gene) for all treatments (Figure 4.20). Average cycle threshold (C_T) value for housekeeping gene was 22, while no C_T values were obtained for *pcpB* and *TftD* genes in the treatment samples. Thus quantification of the gene expression was not possible for PCP degrading genes in phase 1. This may indicate that bacterial genes coding for PCP degrading enzymes were not expressed or expressed below detection limits under the given growth conditions. Another explanation may be that bacteria utilized NB as an energy source instead of PCP and hence did not express genes for PCP degradation. Changes in pH which may have resulted in a decrease in the average bacterial counts over time (Figure 4.4), may also have affected the expression of genes for PCP degradation by these bacteria.



Figure 4.20 Gel electrophoresis of RT-PCR products of treatments 1 and 2 of phase 1 (without air sparging) on day 21.

Note: Presence of only 16S housekeeping gene (indicated by blue arrow) and absence of *pcpB* gene. Lanes 1, 2 and 3 = 16S gene in day 21_TRT 1. rep 1, rep 2 and rep 3; Lanes 4, 5 and 6 = 16S gene in day 21_TRT 2. rep 1, rep 2 and rep 3; Lanes 7, 8 and 9 = *pcpB* gene in day 21_TRT 1.rep 1, rep 2 and rep 3; Lanes 10, 11 and 12 = *pcpB* gene in day 21_TRT 2.rep 1, rep 2 and rep 3; Lane 13= 1 Kb plus ladder.

Phase 2 (with air sparging)

RNA quality

RNA extracted from positive controls (pure cultures of *S. chlorophenolicum* and *B. cepacia*) and groundwater treatments was of good quality. Presence of distinct 16S rRNA and 23S rRNA bands while absence of genomic DNA bands on the 1.5% agarose gel indicated good quality of extracted RNA (Figures 4.21 and 4.22 respectively). RNA samples showed purity ratio A260/280 of 1.6 to 2.0 and A260/230 ratio of 1.2 to 1.8 on NanoDrop™ spectrophotometer.

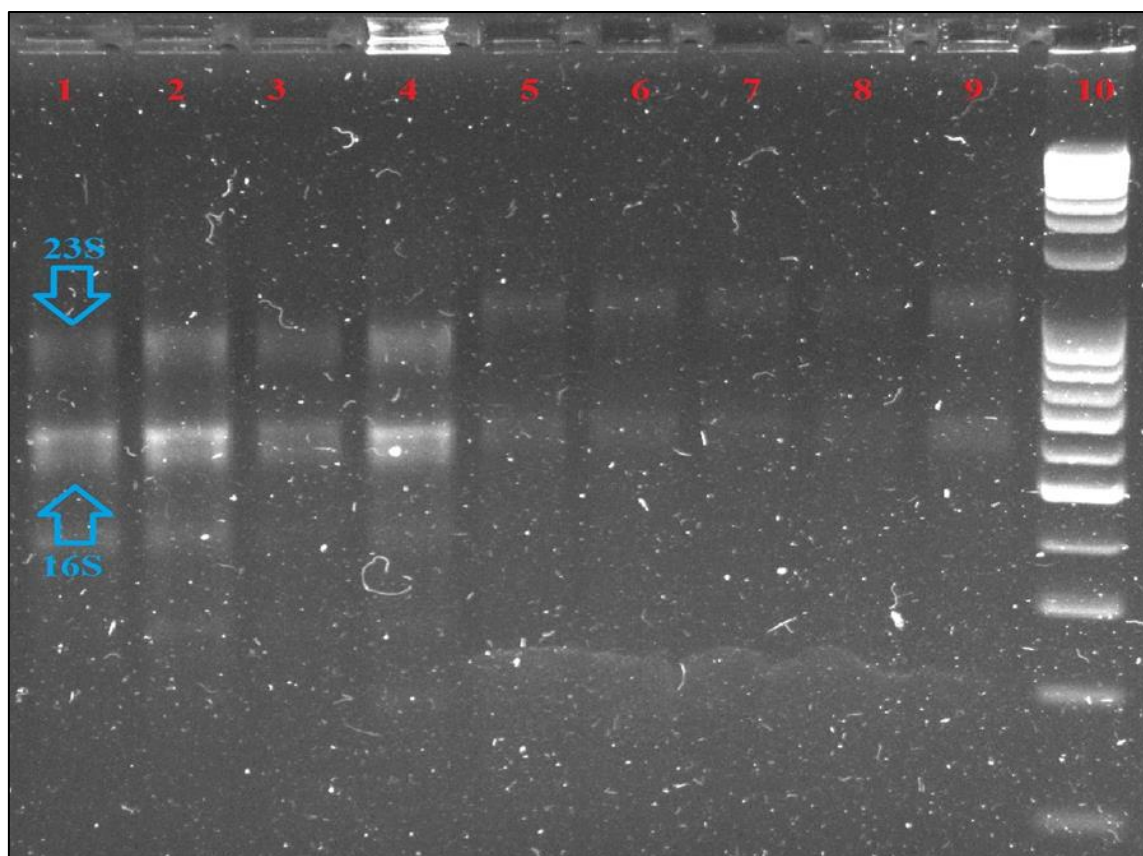


Figure 4.21 RNA extracted from phase 2 (with air sparging) positive controls (pure cultures of *S. chlorophenolicum* and *B. cepacia*).

Note: Lane 1 = *S. chlorophenolicum* at 10 ppm PCP; Lane 2 = *S. chlorophenolicum* at 1 ppm PCP; Lane 3 = *B. cepacia* at 10 ppm PCP; Lane 4 = *B. cepacia* at 1 ppm PCP; Lane 10 = 1 Kb ladder. Note: Blue color arrows indicate 16S and 23S rRNA bands.

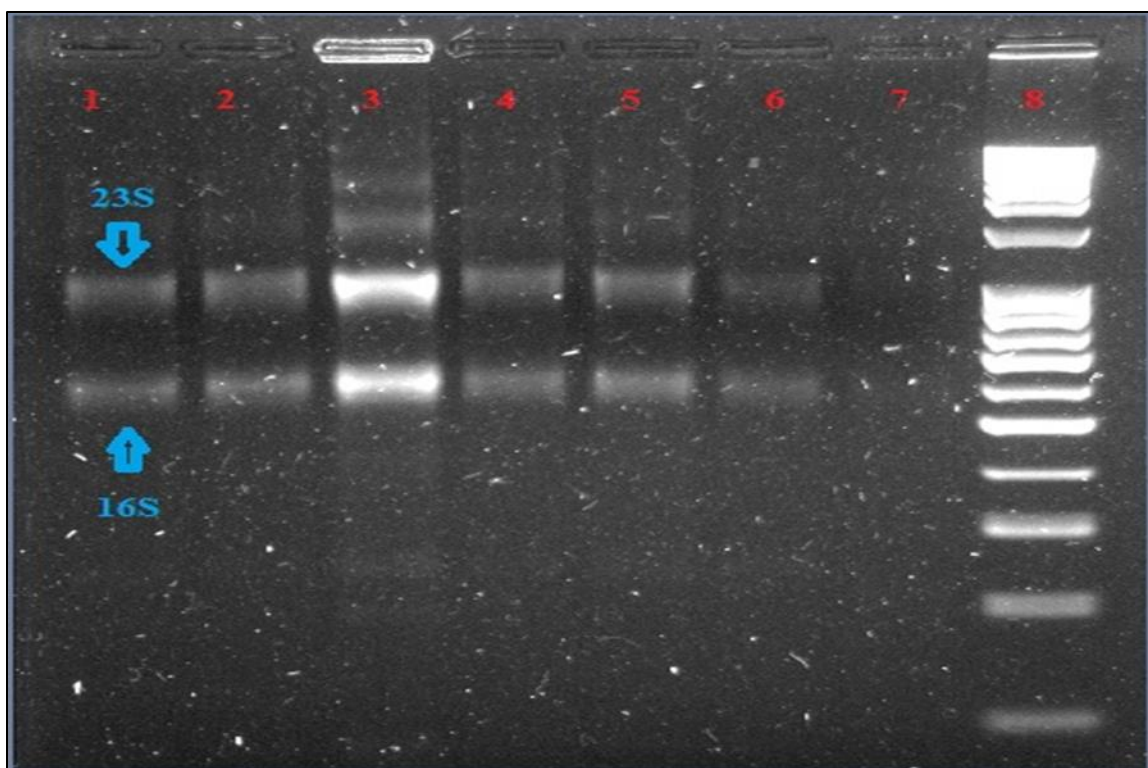


Figure 4.22 RNA extracted from phase 2 (with air sparging) groundwater treatment cultures on day 36.

Note: Lanes 1, 2 and 3 = Day 36_TRT 1. rep 1, rep 2 and rep 3; Lane 4, 5 and 6 = Day 36_TRT 2. rep 1, rep 2 and rep 3; Lane 8 = 1Kb plus ladder. Blue colored arrows indicate presence of 16S and 23S rRNA bands.

Verification of gene specific primers

Amplification of cDNA from pure cultures of *S. chlorophenolicum* and *B. cepacia* (positive controls for phase 2) by *pcpB* and *TftD* specific primers showed bands for amplification products of expected size 192bp and 185bp, respectively (Figure 4.23). Thus, these two genes were expressed in positive controls respectively and verified the suitability of the primers for detection of these genes. Amplification of DNA and cDNA from groundwater treatments by 16S rRNA gene specific primers and presence of bands

for the amplified products at 173bp indicated presence of bacterial RNA and suitability of this gene to be used as a housekeeping gene for RT-PCR (Figures 4.23 and 4.24).

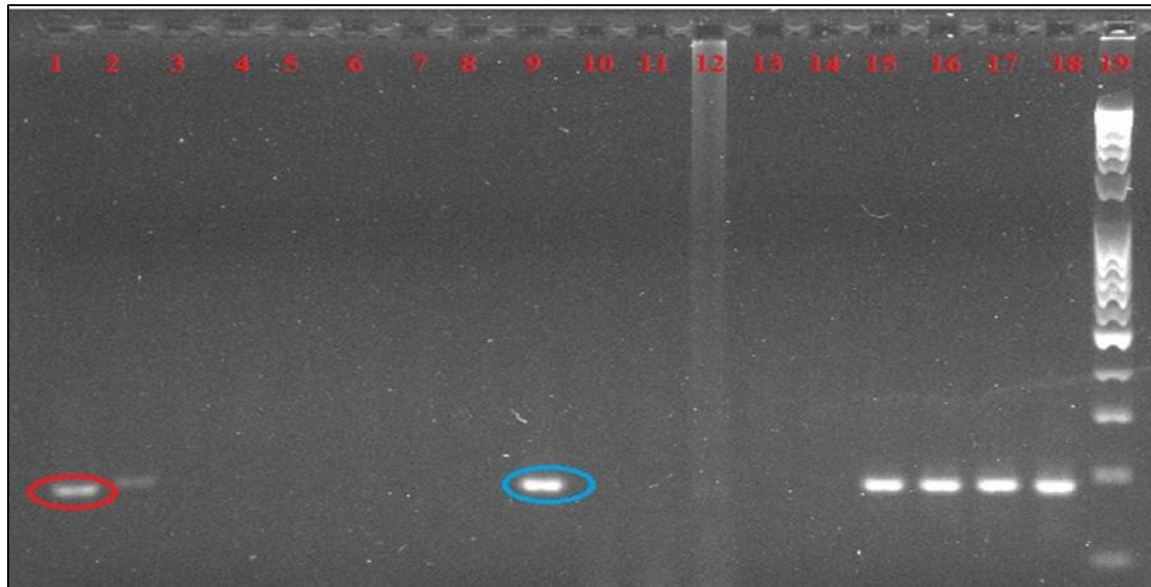


Figure 4.23 Amplification of cDNA samples of phase 2 (with air sparging) positive controls (*S. chlorophenolicum* and *B. cepacia*) and groundwater treatment cultures.

Note: Lane 1 = *pcpB* gene in *S. chlorophenolicum* (in red circle); Lane 9 = TftD gene in *B. cepacia* (in blue circle), Lane 15, 16, 17 and 18 = 16S housekeeping gene in TRTs 1, 2, 3 and 4 respectively; Lane 19 = 1 Kb plus ladder.

Presence of a band for the amplification product of the *pcpB* gene (specific to *S. chlorophenolicum*) of 192bp in extracted DNA from treatment 2 (Miracle Gro™ + *S. chlorophenolicum*) indicated presence of the PCP degrading gene of *S. chlorophenolicum* (Figure 4.24, Lane 10). Presence of a band of 185bp for the amplification product of TftD (specific to *B. cepacia*) gene in extracted DNA from treatment 3 (Miracle Gro™ + *B. cepacia*) and treatment 4 (Miracle Gro™ + *B. cepacia* + *S. chlorophenolicum*) indicated presence of PCP degrading gene of *B. cepacia* in both treatments (Figure 4.24, Lanes 14,

15). Thus it may be concluded that the designed primers were specific for genes *pcpB* and TftD and are suitable for determination of mRNA expression of these genes.

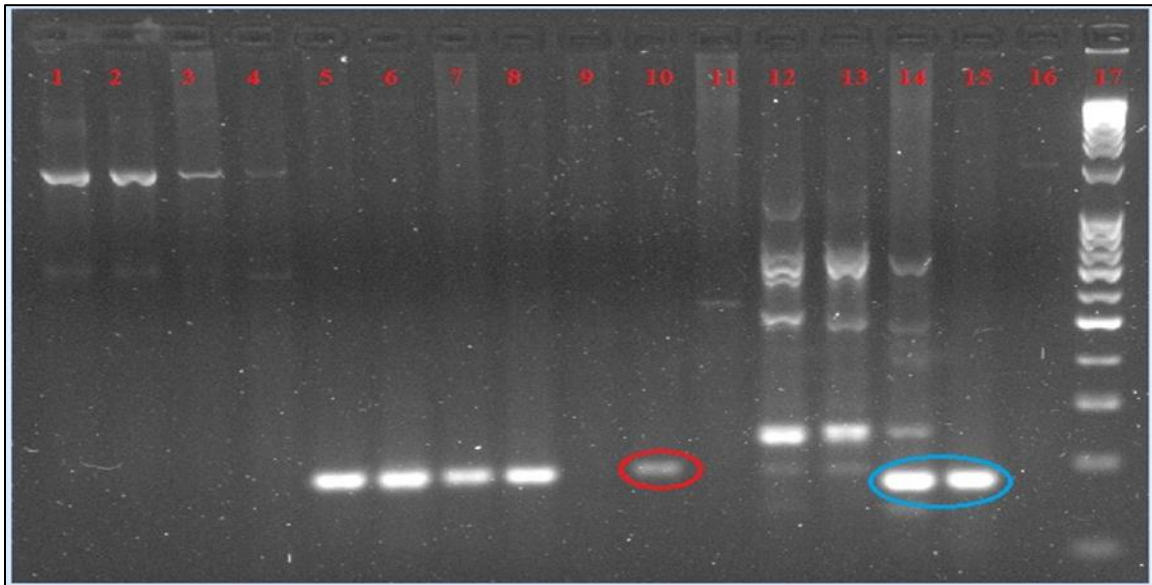


Figure 4.24 Amplification of DNA of phase 2 (with air sparging) groundwater treatments using gene specific primers.

Note: Lanes 1, 2, 3 and 4 = 16S gene (1500bp) in TRT 1, 2, 3 and 4; Lanes 5, 6, 7 and 8 = 16S housekeeping gene (173bp) in TRT 1, 2, 3 and 4; Lane 10 = presence of *pcpB* gene in TRT 2 (in red circle); Lanes 14, 15 = presence of TftD in TRT 3 and TRT 4 respectively (in blue circle); Lane 17= 1 Kb plus ladder.

Quantitation of gene expression using RT-PCR

RT-PCR was performed on positive controls (pure cultures of *B. cepacia* and *S. chlorophenolicum*) and all groundwater treatments on sampling days 0, 36 and 72 to study expression of TftD and *pcpB* genes. Results indicated that both genes were expressed in the respective positive control and all groundwater treatments. Average C_T values obtained for TftD and *pcpB* genes were 26 and 33 cycles in positive controls *B. cepacia* and *S. chlorophenolicum*, respectively, indicating strong gene expression in pure cultures. Average C_T values obtained for TftD gene for all groundwater treatments were

33-34 cycles indicating moderate levels of gene expression, while average C_T values obtained for the housekeeping gene ranged from 22-23 cycles. Average C_T values obtained for *pcpB* gene were 35-36 cycles indicating relatively low gene expression in groundwater treatments. Gene expressions of both genes (*pcpB* and *TftD*) varied in all treatments over time (Figures 4.25- 4.27, 4.29-4.31 respectively).

Chlorophenol 4-monooxygenase (TftD) gene expression

On day 0, the relative fold expression of *TftD* (specific to *B. cepacia*) was highest in treatment 4 (Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*) compared to treatment 1 (Figure 4.25). On day 36, the relative fold expression of *TftD* increased by approximately 7 fold in treatments 2 (Miracle Gro™ + *S. chlorophenolicum*) and treatment 3 (Miracle Gro™ + *B. cepacia*) while, it did not change in treatment 4 compared to day 0 (Figure 4.26). On day 72, the relative fold expression of *TftD* increased (10.7 fold and highest) in treatment 3, while it decreased (5 fold) in treatment 2 and unchanged in treatment 4 compared to day 36 (Figure 4.27).

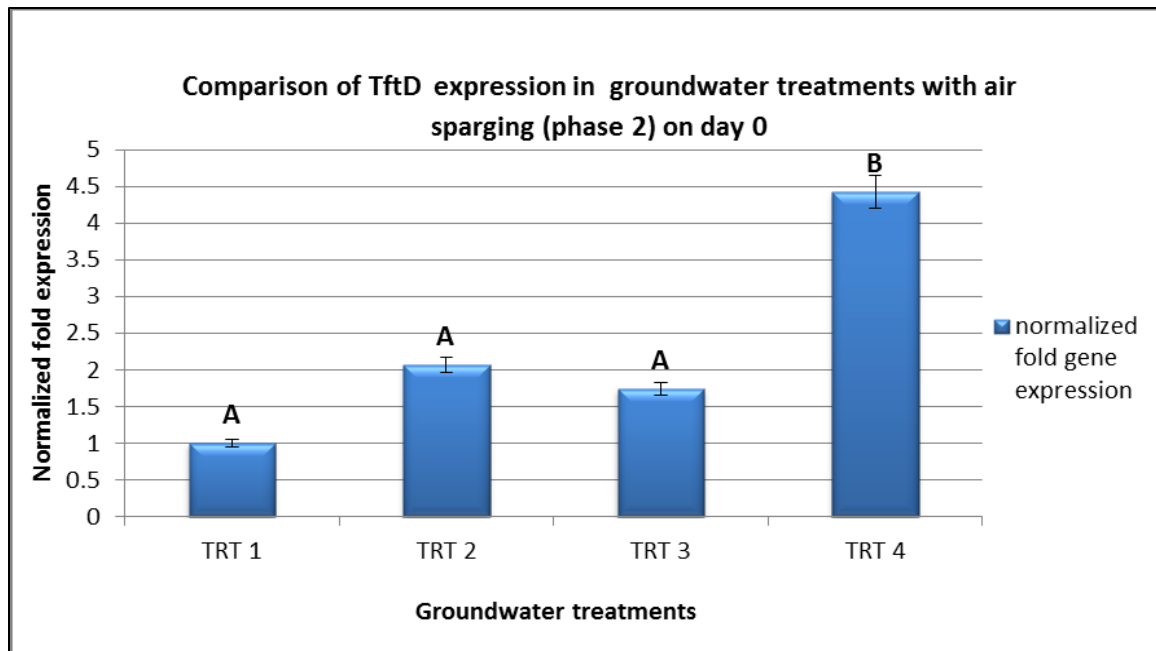


Figure 4.25 Comparison of TftD gene expression among groundwater treatments with air sparging (phase 2) on day 0.

Note: Treatments: TRT 1 = with Miracle Gro™, TRT 2 = Miracle Gro™ + *S. chlorophenolicum*, TRT 3 = Miracle Gro™ + *B. cepacia* and TRT 4= Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*. There were three replicates per treatment. Letters A and B indicate significant differences between TftD expression in groundwater treatments. Statistical Values= F critical: 9.7, F value= 208.17 and P value= 0.0048.

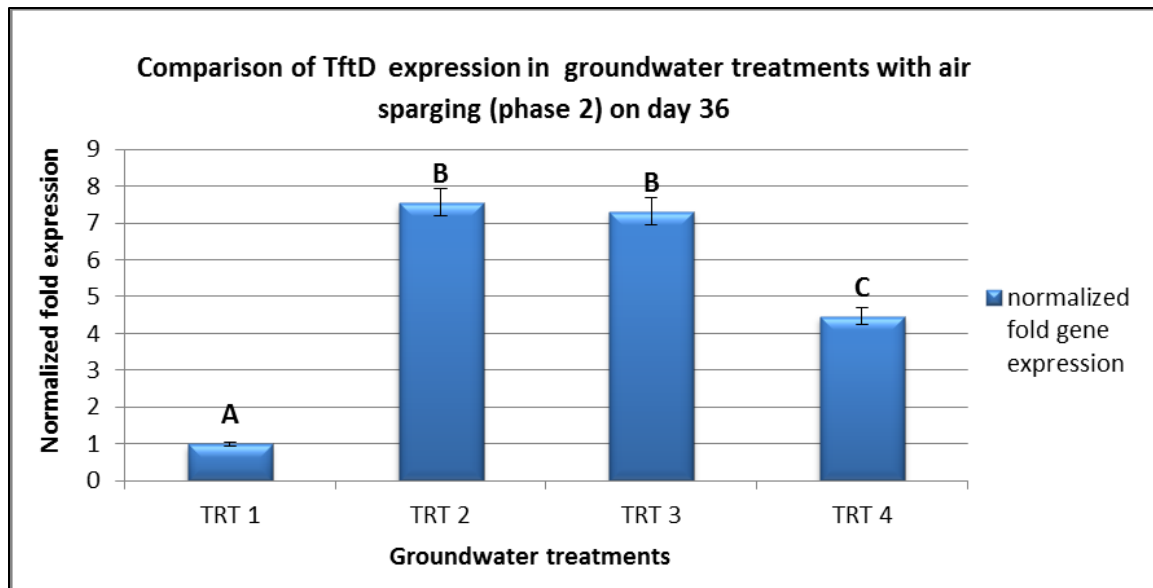


Figure 4.26 Comparison of TftD gene expression in groundwater treatments with air sparging (phase 2) on day 36.

Note: Treatments: TRT 1 = with Miracle Gro™, TRT 2 = Miracle Gro™ + *S. chlorophenolicum*, TRT 3 = Miracle Gro™ + *B. cepacia* and TRT 4= Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*. There were three replicates per treatment. Letters A, B and C indicate significant differences between TftD expressions in groundwater treatments. Statistical Values= $\alpha = 0.05$, F critical: 9.7, F value= 418.72 and P value= 0.0024.

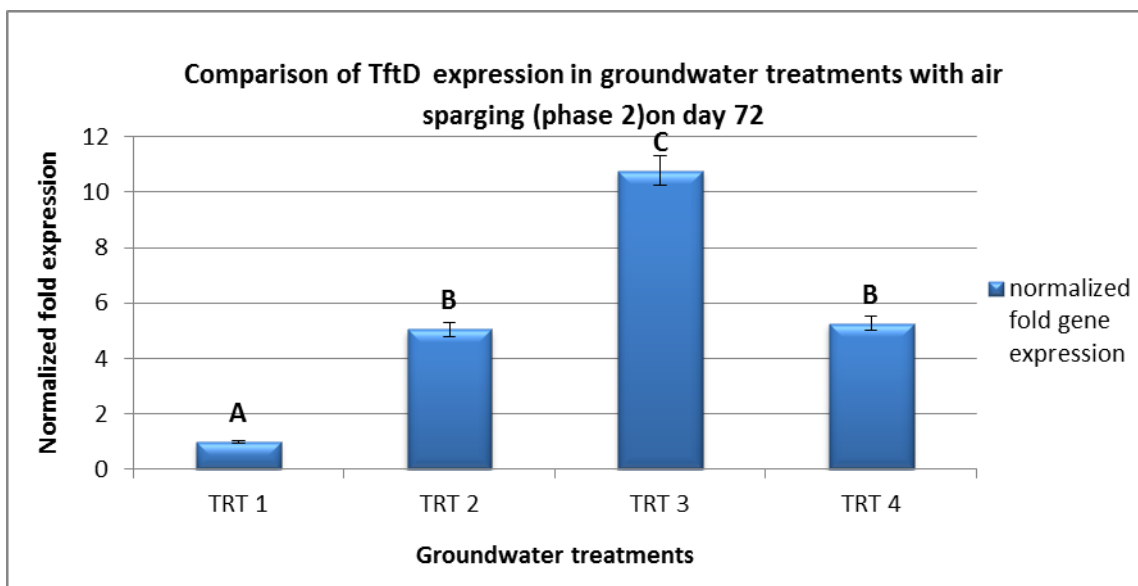


Figure 4.27 Comparison of TftD gene expression between groundwater treatments with air sparging (phase 2) on day 72.

Note: Treatments: TRT 1 = with Miracle Gro™, TRT 2 = Miracle Gro™ + *S. chlorophenolicum*, TRT 3 = Miracle Gro™ + *B. cepacia* and TRT 4= Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*. There were three replicates per treatment. Letters A, B and C indicate significant differences between TftD expressions in groundwater treatments. Statistical Values= $\alpha = 0.05$, F critical: 9.7, F value= 333.15 and P value= 0.0030.

All treatments contained *B. cepacia* because it was identified as the dominant bacteria in the indigenous groundwater bacterial community (Figure 4.11), which may explain the expression of TftD gene in all treatments (un-inoculated (treatment 1) as well as inoculated with *B. cepacia* pure culture (treatment 3)). Overall treatment 3 showed an increasing gene expression for TftD (Figure 4.28), which is not unexpected as treatment 3 was inoculated with pure culture of *B. cepacia* ATCC® 53867. Thus this may correlate to the increased PCP degradation in treatment 3 (Figures 4.8 and 4.13 respectively). Though treatment 4 was inoculated with *B. cepacia* along with *S. chlorophenolicum*, RNA expression of TftD did not show a relative fold increase over time as observed in

treatment 3 (Figure 4.28). This may indicate that interaction between *S. chlorophenolicum* and *B. cepacia* has an antagonistic effect which may also have resulted in lower number of PCP tolerant bacteria (Figure 4.8) than other treatments and no significant decrease in average PCP concentration over time (Figure 4.13) (Slater and Lovatt 1984 and Wijngaard et al. 1993).

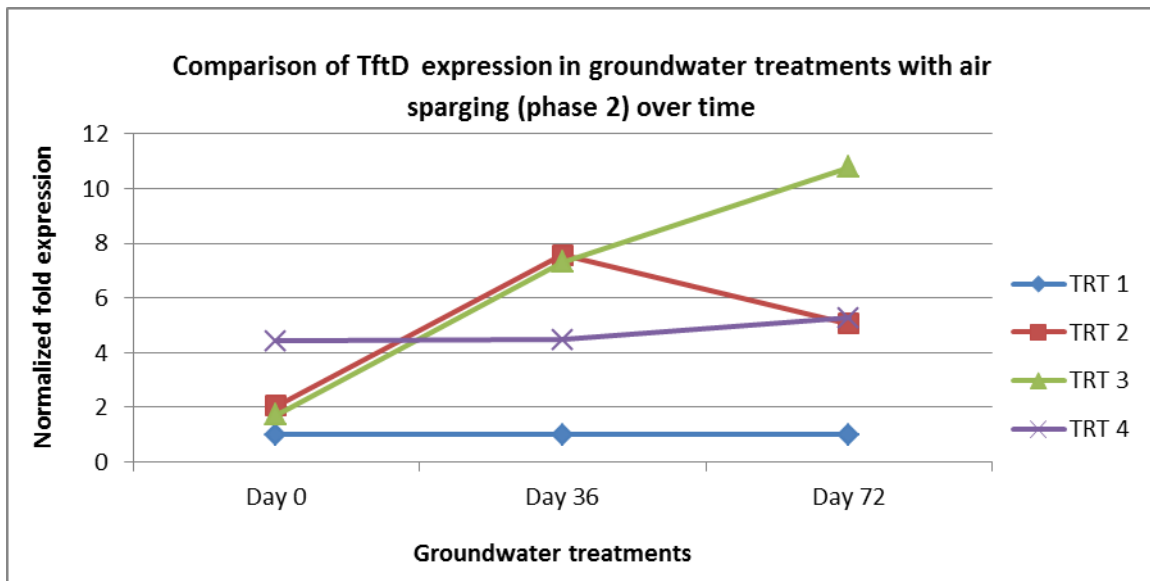


Figure 4.28 Comparison of TftD gene expression in groundwater treatments with air sparging (phase 2) over sampling times day 0, 36 and 72.

Note: Treatments: TRT 1 = with Miracle Gro™, TRT 2 = Miracle Gro™ + *S. chlorophenolicum*, TRT 3 = Miracle Gro™ + *B. cepacia* and TRT 4 = Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*. There were three replicates per treatment.

Pentachlorophenol-4-monooxygenase (pcpB) gene expression

Relative fold expressions of *pcpB* (specific to *S. chlorophenolicum*) in individual groundwater treatments were significantly different from each other on sampling days 0, 36 and 72 (Figures 4.29, 4.30 and 4.31 respectively). On day 0 expression of *pcpB* gene in treatment 2 (Miracle Gro™ + *S. chlorophenolicum*) was decreased by 2.2 fold as

compared to treatment 1 (without added inoculums), while no gene expression was observed in treatments 3 (Miracle Gro™ + *B. cepacia*) and 4 (Miracle Gro™ + *B. cepacia* + *S. chlorophenolicum*) (Figure 4.29). Thus very little if any gene expression present on day 0 resulted from the low PCP tolerant bacterial counts (Figure 4.8).

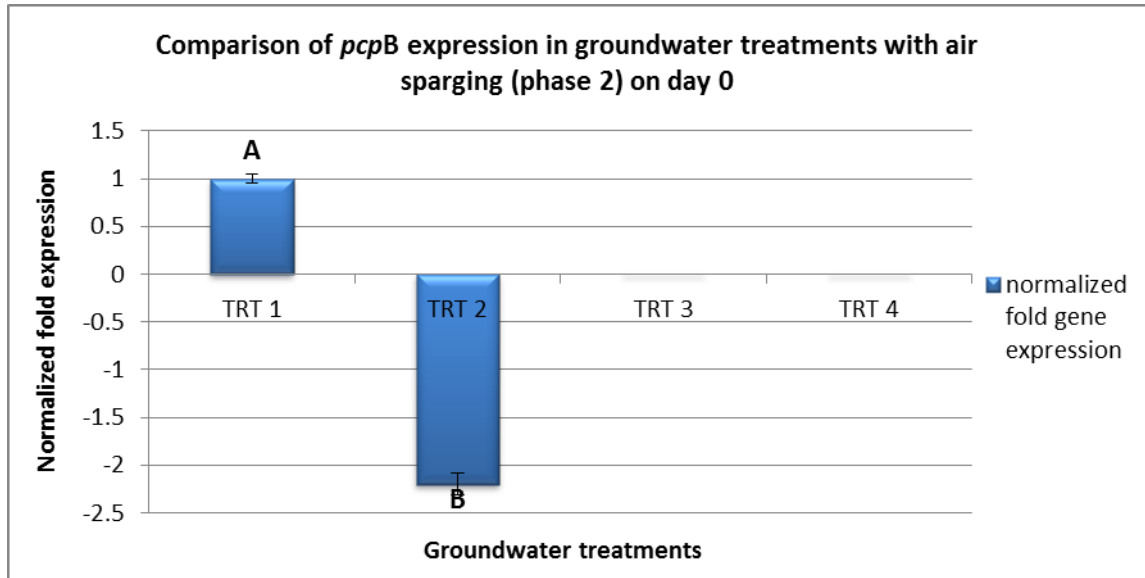


Figure 4.29 Comparison of *pcpB* (pentachlorophenol-4-monooxygenase) gene expression in groundwater treatments with air sparging (phase 2) on day 0.

Notes: Treatments: TRT 1 = with Miracle Gro™, TRT 2 = Miracle Gro™ + *S. chlorophenolicum*, TRT 3 = Miracle Gro™ + *B. cepacia* and TRT 4 = Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*. There were three replicates per treatment. Statistical Values: $\alpha = 0.05$, F critical: 9.7, F value= 270.75 and P value= 0.0038. Note: no gene expression was observed in TRT 3 and TRT 4 due to low PCP tolerant bacteria on day 0.

In contrast, on day 36, relative fold expressions of *pcpB* gene increased in treatments 2, 3 and 4 with highest (7 fold) in treatment 4. This may have been the result of increased PCP tolerant bacteria compared to day 0 (Figures 4.30 and 4.8 respectively).

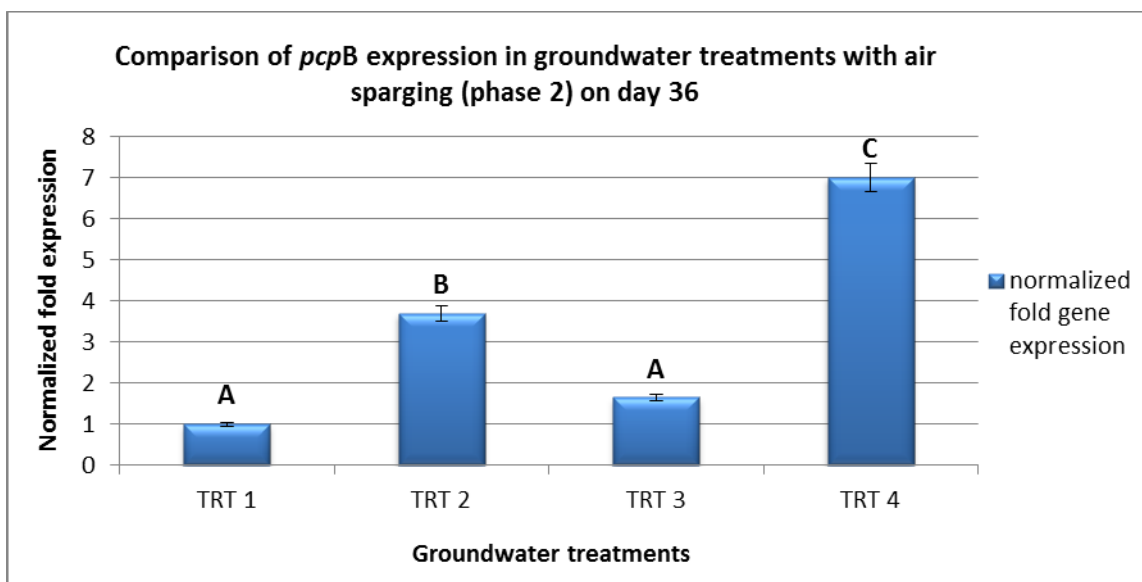


Figure 4.30 Comparison of *pcpB* (pentachlorophenol-4-monooxygenase) gene expression in groundwater treatments with air sparging (phase 2) on day 36.

Note: Treatments: TRT 1 = with Miracle Gro™, TRT 2 = Miracle Gro™ + *S. chlorophenolicum*, TRT 3 = Miracle Gro™ + *B. cepacia* and TRT 4 = Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*. There were three replicates per treatment. Statistical Values: $\alpha = 0.05$, F critical: 9.7, F value= 247.25 and P value= 0.004.

On day 72, treatment 2 showed an increased (7 fold and highest) relative fold gene expression of *pcpB* gene while it was unchanged in treatment 3 and decreased (5 fold) in treatment 4 as compared to day 36 (Figure 4.31). In addition, treatment 2 also showed an increasing pattern of relative fold expression of *pcpB* gene over time (Figure 4.32). This was expected as treatment 2 was inoculated with *S. chlorophenolicum* strain L-1 ATCC® 53874. This may indicate that *S. chlorophenolicum* population in treatment 2 showed increase in growth and PCP degradation (Figures 4.8 and 4.13 respectively). Treatment 4 showed a decrease in the relative fold expression of *pcpB* gene after day 36 (Figures 4.30 and 4.31 respectively) which may also correlate with lower PCP tolerant bacteria (Figure 4.8) and no significant decrease in PCP concentration over time (Figure

4.13). Therefore it is possible that an antagonistic effect exists between *S. chlorophenolicum* and *B. cepacia* on expression of *pcpB* gene (Slater and Lovatt 1984 and Wijngaard et al. 1993).

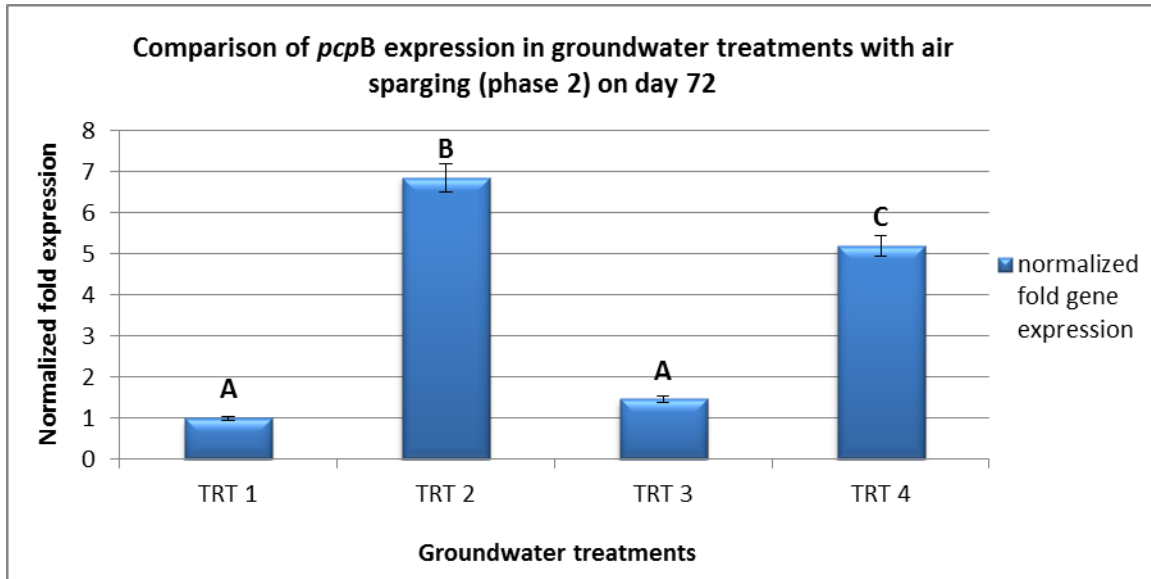


Figure 4.31 Comparison of *pcpB* (pentachlorophenol-4-monooxygenase) gene expression in groundwater treatments with air sparging (phase 2) on day 72.

Note: Treatments: TRT 1 = with Miracle Gro™, TRT 2 = Miracle Gro™ + *S. chlorophenolicum*, TRT 3 = Miracle Gro™ + *B. cepacia* and TRT 4 = Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*. There were three replicates per treatment. Statistical Values: $\alpha = 0.05$, F critical: 9.7, F value= 310.38 and P value= 0.0032.

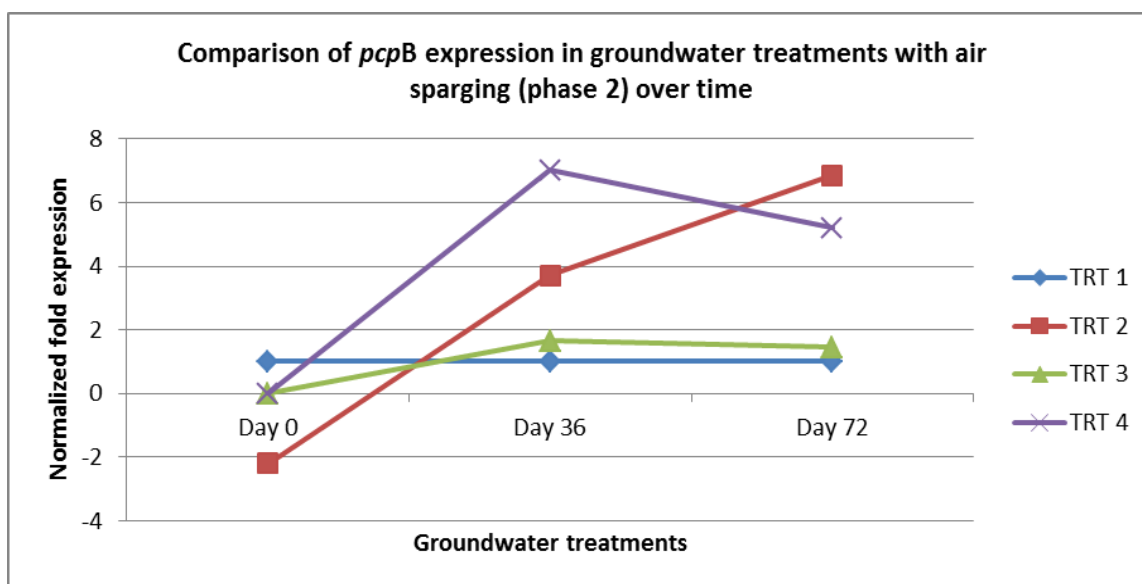


Figure 4.32 Comparison of *pcpB* (pentachlorophenol-4-monooxygenase) gene expression in groundwater treatments with air sparging (phase 2) over sampling days 0, 36 and 72.

Note: Treatments: TRT 1 = with Miracle Gro™, TRT 2 = Miracle Gro™ + *S. chlorophenolicum*, TRT 3 = Miracle Gro™ + *B. cepacia* and TRT 4 = Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*. There were three replicates per treatment.

Correlation: gene expression and PCP degradation

A strong negative correlation ($r = -0.8139$) resulted between normalized relative fold gene expression of *TftD* and average PCP concentration data (Figure 4.33), while a weak negative correlation ($r = -0.4105$) resulted between normalized relative fold gene expression of *pcpB* gene and average PCP concentration data was observed (Figure 4.34). This indicated that an increase in the relative fold gene expression of these genes was correlated with a decrease in average PCP concentration over time (Figures 4.33 and 4.34 respectively). Treatment 3 (Miracle Gro™ + *B. cepacia*) which had the highest increase in relative fold *TftD* gene expression, showed the maximum PCP degradation of 49% in comparison to treatment 1 (Figures 4.33 and 4.13 respectively).

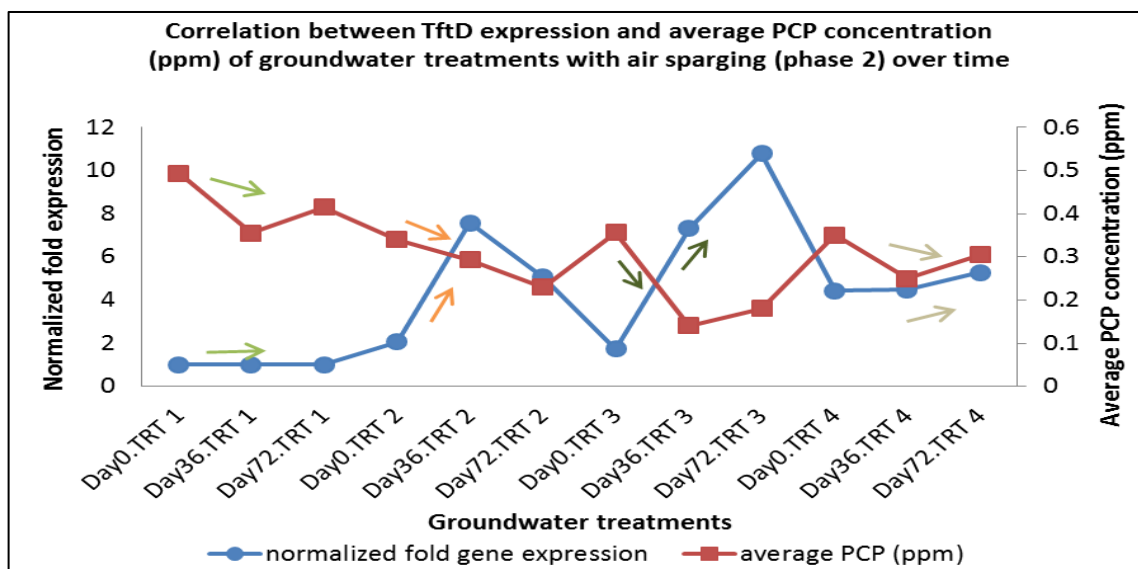


Figure 4.33 Correlation between TftD expression and average PCP concentration (ppm) of groundwater treatments with air sparging (phase 2) over sampling days 0, 36 and 72.

Note: Treatments: TRT 1 = with Miracle Gro™, TRT 2 = Miracle Gro™ + *S. chlorophenolicum*, TRT 3 = Miracle Gro™ + *B. cepacia* and TRT 4 = Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*. There were three replicates per treatment. Correlation coefficient (r) = -0.8139.

Also treatment 2 (Miracle Gro™ + *S. chlorophenolicum*) which had the highest increase in relative fold *pcpB* gene expression, showed significant decrease of 32% in PCP concentration in comparison to treatment 1 (Figures 4.34 and 4.13 respectively). Treatment 4 (Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*) which had no change in relative fold expression of TftD and decrease in relative fold expression of *pcpB* after day 36, did not show a significant reduction in PCP concentration over time (Figures 4.33, 4.34 and 4.13 respectively). Thus this indicated that significant decreases in the average PCP concentration in treatments 2 and 3 over time was most likely caused by an increased expression of *pcpB* and TftD genes by the PCP degrading bacteria respectively.

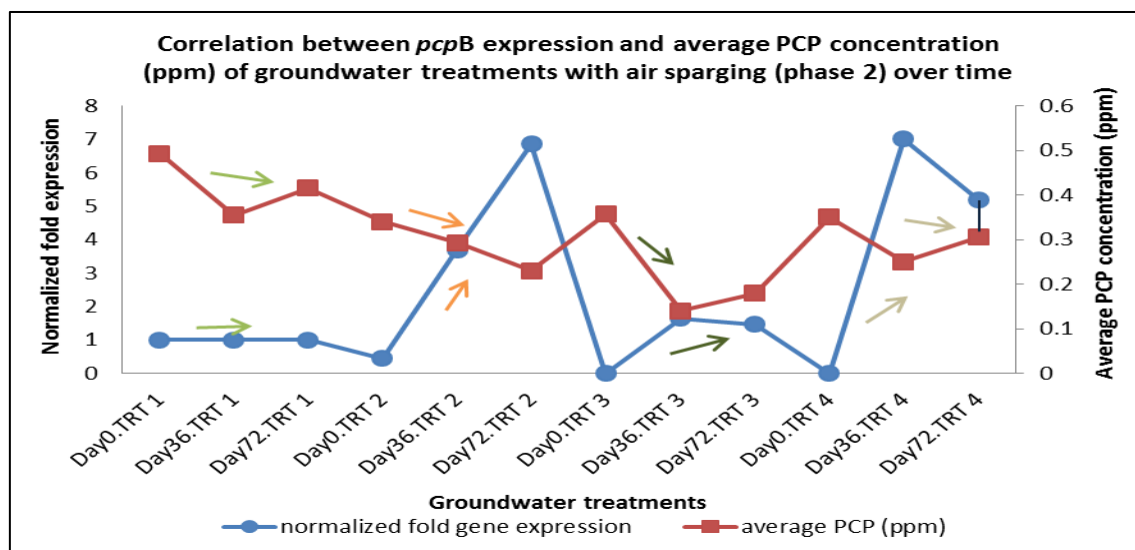


Figure 4.34 Correlation between *pcpB* expression and average PCP concentration (ppm) of groundwater treatments with air sparging (phase 2) over sampling days 0, 36 and 72.

Note: Treatments: TRT 1 = with Miracle Gro™, TRT 2 = Miracle Gro™ + *S. chlorophenolicum*, TRT 3 = Miracle Gro™ + *B. cepacia* and TRT 4 = Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*. There were three replicates per treatment. Correlation coefficient (r) = -0.4105.

Comparison: positive controls and groundwater treatments

The relative fold gene expressions of both *TftD* and *pcpB* genes in all treatments were expressed in low levels when compared with expressions of these genes in respective positive controls. Pure cultures of *B. cepacia* showed 75 fold higher gene expression of *TftD* gene than treatment 1 (without added inoculums) (Figure 4.35). While pure cultures of *S. chlorophenolicum* showed 43 fold higher gene expression of *pcpB* gene than treatment 1 (Figure 4.36).

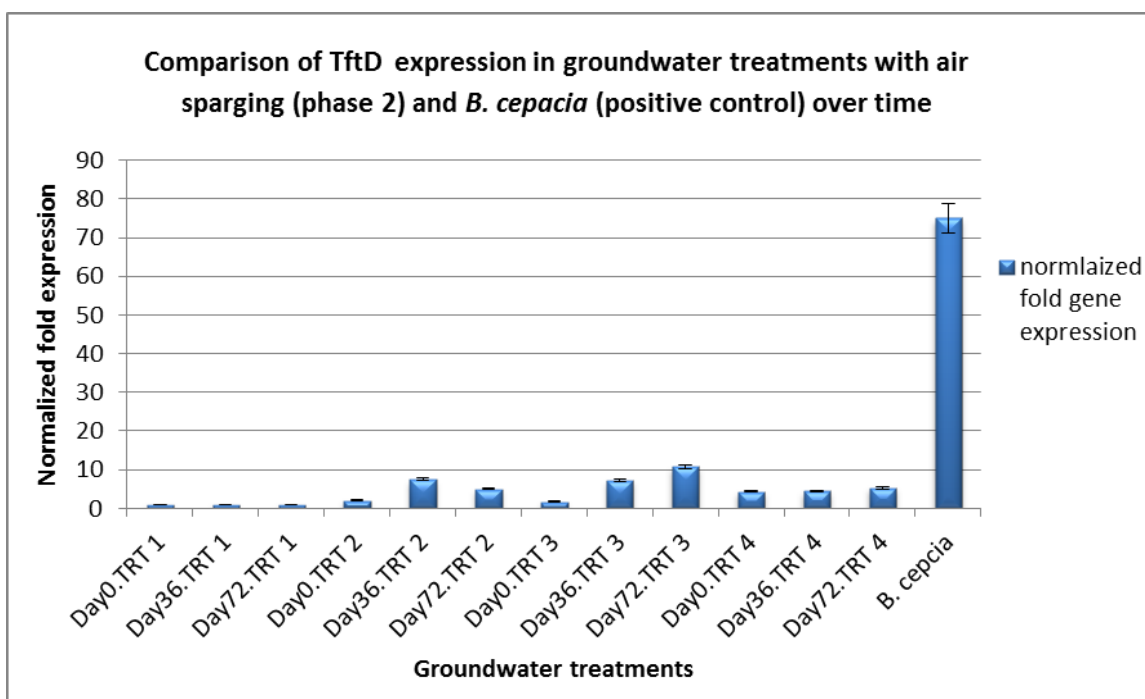


Figure 4.35 Comparison of TftD gene expression in groundwater treatments with air sparging (phase 2) and *B. cepacia* (positive control) over sampling days 0, 36 and 72.

Note: Treatments: TRT 1 = with Miracle Gro™, TRT 2 = Miracle Gro™ + *S. chlorophenolicum*, TRT 3 = Miracle Gro™ + *B. cepacia* and TRT 4 = Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*. There were three replicates per treatment.

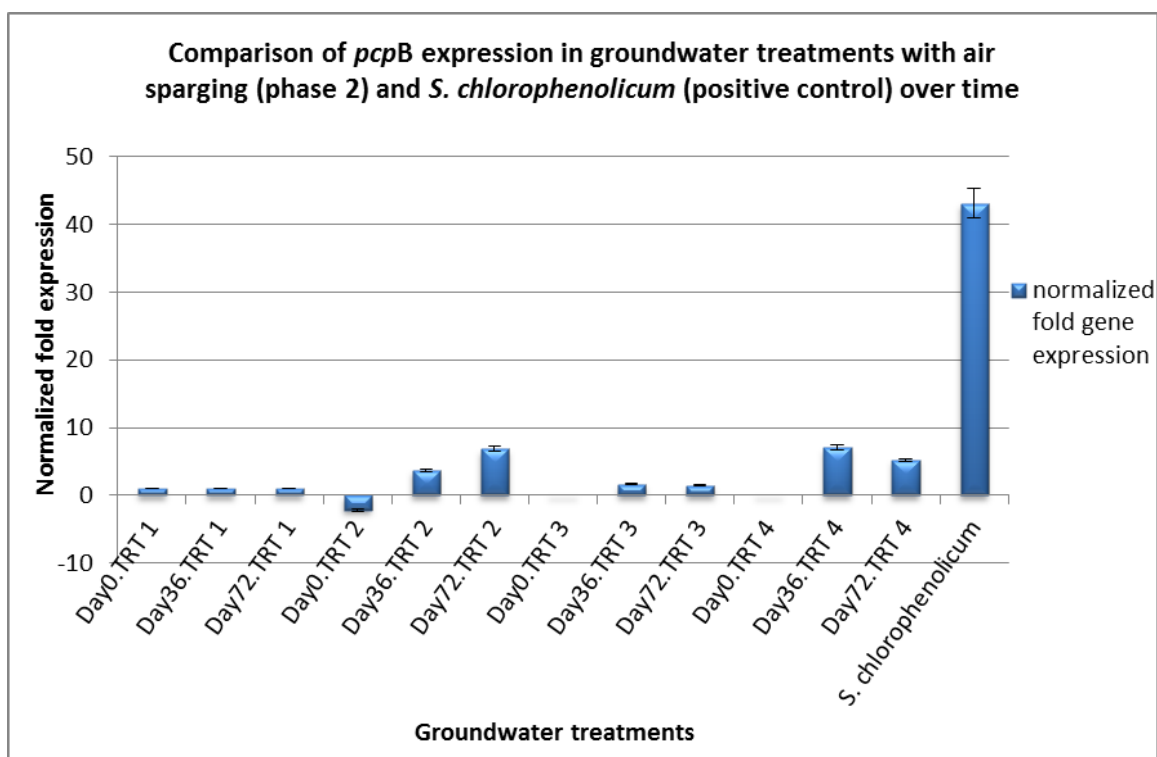


Figure 4.36 Comparison of *pcpB* expression in groundwater treatments with air sparging (phase 2) and *S. chlorophenolicum* (positive control) over sampling days 0, 36 and 72.

Note: Treatments: TRT 1 = with Miracle Gro™, TRT 2 = Miracle Gro™ + *S. chlorophenolicum*, TRT 3 = Miracle Gro™ + *B. cepacia* and TRT 4 = Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*. There were three replicates per treatment.

Comparison: phase 1 and phase 2

Relative fold gene expression of PCP degrading genes was not observed in phase 1 (without air sparging) compared to phase 2 (with air sparging). This may be the effect of Miracle Gro™ and air sparging stimulating bacterial growth and PCP degradation activity in phase 2. Inoculation of known PCP degrading bacteria (*S. chlorophenolicum* and *B. cepacia*) to the indigenous groundwater community (treatments 2, 3 and 4 of phase 2) may also have resulted in increased expression of PCP degrading enzymes (PCP-4-monooxygenase and chlorophenol 4-monooxygenase) and hence degradation of

PCP in phase 2. In addition to this, the use of ATCC 1687 medium for culturing of phase 2 groundwater treatments may have stimulated the expression of PCP degrading genes, as it has very low amounts of sodium mono-glutamate as a sole source of energy and hence bacteria may have degraded PCP as a source of energy. In contrast, phase 1 culturing was done in NB which is a rich source of energy for bacteria. Thus bacteria may have utilized NB as energy source instead of degrading PCP and hence no expression of PCP degrading genes was observed in phase 1.

In summary, the decrease in average PCP concentration over time in phase 1 may have been due to the volatilization of PCP from groundwater or experimental error. While in phase 2 decreases in average PCP concentration over time was most likely the effect of PCP degradation by bacteria.

CHAPTER V

CONCLUSIONS

Bacterial enumeration and correlation with pH

There were fewer total and PCP tolerant bacteria in phase 1 (without air sparging) than in phase 2 (with air sparging). In phase 1, the pH varied within treatments but the average PCP tolerant bacteria decreased over time indicating no correlation ($r = 0.019$). In contrast, in phase 2 a strong positive correlation ($r = 0.9176$) indicated that as the pH increased from acidic to optimal, PCP tolerant bacteria also increased over time. Thus optimum pH and air sparging resulted in a higher PCP tolerant bacterial count which may be beneficial for degradation of PCP in the groundwater.

Identification of groundwater bacterial community

Approximately 62% of the groundwater bacterial community was composed of known PCP degrading bacteria such as *Burkholderia* sp. (35%), *S. chlorophenolicum* sp. (6%), *Bacillus cereus* sp. (18%) and *Pseudomonas* sp. (3%). *Burkholderia* sp. was identified as a dominant bacteria present in the groundwater. The presence of these PCP degrading bacteria in the indigenous groundwater bacterial community could be beneficial for the bioremediation of PCP contaminated groundwater.

PCP concentration in the groundwater

In both phase 1 and phase 2, the average PCP concentrations in all treatments over time were higher than the EPA recommended maximum contaminant load (MCL) of 1 ppb for PCP in the drinking water. In phase 1 (without air sparging), a significant decrease (18%) in the average PCP concentration in treatment 2 (with Miracle Gro™) compared to treatment 1 (without Mircle Gro™) was most likely caused by the effect of the addition of Miracle Gro™. In phase 2 (Miracle Gro™ + air sparging), treatments 2 and 3 inoculated with a single inoculum of known PCP degrading bacteria (*S. chlorophenolicum* and *B. cepacia* respectively) showed higher PCP degradation (32% and 49% respectively) than indigenous (treatment 1, 16%) and inoculated mixed culture (treatment 4, 13%). Although there were reductions in PCP concentrations in all treatments, the remaining PCP in the groundwater was not below the MCL (1ppb).

Gene expression of PCP degrading enzymes

In phase 1 (without air sparging), there was no gene expression obtained for TftD (specific to *B. cepacia*) and *pcpB* (specific to *S. chlorophenolicum*). In phase 2 (with air sparging), both genes were expressed but expression varied in all treatments over time. Gene expressions were higher in treatments amended with a single inoculum of known PCP degrading bacteria (*B. cepacia* and *S. chlorophenolicum*) as compared to indigenous and mixed culture inoculated treatments. Expression of these genes in the un-inoculated treatment was most likely due to the presence of *B. cepacia* and *S. chlorophenolicum* in the indigenous groundwater bacterial community. RNA expression levels of TftD and *pcpB* genes in groundwater treatments were low compared to pure cultures of *B. cepacia* and *S. chlorophenolicum* respectively. Thus bio-augmentation with known PCP

degrading bacteria to the indigenous bacterial community and air sparging may have been the reason for observed expression of PCP degrading enzymes in phase 2 compared to phase 1.

Correlation between gene expression and PCP degradation

In phase 1 (without air sparging) there was no correlation between PCP degradation and gene expression for PCP degrading enzymes (TftD and *pcpB*). In phase 2 (with air sparging), negative correlations ($r = -0.81$ and -0.41) were observed in single culture inoculated treatments suggesting that relative fold increase in the gene expression of these PCP degrading enzymes correlated to a decrease in PCP concentration. Thus it may be concluded that the decrease in PCP observed in phase 2 was due to the bacterial degradation of PCP which was higher in treatments with single inoculums of selective PCP degrading bacteria compared to un-inoculated (indigenous) treatment. Indigenous bacterial community may have contributed to gene expression and PCP degradation in single inoculum treatments (2, 3) because many (62%) indigenous bacteria were identified as PCP degraders with *B. cepacia* being dominant species.

Comparison: indigenous and bio-augmented PCP degrading bacteria

Treatments inoculated with selected known PCP degrading bacteria (*B. cepacia* and *S. chlorophenolicum*) showed higher gene expression for PCP degrading enzymes (chlorophenol 4-monooxygenase (TftD) and pentachlorophenol-4-monooxygenase (*pcpB*) respectively) and degradation of PCP than the indigenous bacterial treatment. Treatment augmented with *B. cepacia* showed higher PCP degradation than treatment inoculated with *S. chlorophenolicum*.

Applications in the in-situ bioremediation

In-situ biosparging is an economic and environmental friendly alternative for traditional methods for remediation of PCP contaminated groundwater. Identification of indigenous groundwater bacterial community before beginning of treatment helps to select the known PCP degrading bacteria to be augmented in the groundwater. Improvements in growth conditions such as pH, nutrient and oxygen availability may enhance PCP degradation potential of bacteria. Monitoring gene expression and its correlation with PCP degradation by known PCP degrading bacteria and indigenous bacteria helped to determine which members of the community participate in the PCP degradation. Thus it may be concluded from this study that bio-augmentation of groundwater with known PCP degrading bacteria, amendment of nutrients and air sparging result in an increased degradation of PCP and hence bioremediation of PCP contaminated groundwater. These amendments to the site undergoing air sparging may result in more effective and less time consuming remediation of PCP contaminated groundwater without adding significantly high cost and labor. In addition, bio-augmentation of selective bacteria to the indigenous bacteria may provide a continuous remediation technique compared to other short and long term treatments as these bacteria continue to survive and degrade the contaminant at the site.

Limitations and future studies

Results obtained for degradation of PCP under controlled laboratory conditions may be different from that in environmental conditions due to various factors such as presence of organic matter, bioavailability of PCP, temperature, pH, groundwater flow and changes in bacterial population. Detailed effects of these factors on biodegradation of

PCP must be determined. To make an absolute conclusion about the effect of inoculation of known bacteria on degradation of PCP in groundwater, activity of PCP degrading enzymes should be determined. Interactions such as competitive behavior for the limited substrate, synergistic and antagonistic effect between the known PCP degrading bacteria and indigenous bacteria must be studied to determine the effect of bio-augmentation on the in-situ remediation of PCP in the groundwater. *Burkholderia cepacia* ATCC® 53867 used in this study is a biosafety level 2 organism and hence it cannot be directly added to groundwater though it showed the highest degradation of PCP. Thus the PCP degradation potential of closely related and non-hazardous strains of indigenous *B. cepacia* in the groundwater at PCP contaminated sites should be determined.

REFERENCES

- Agency for Toxic Substances and Disease Registry (ATSDR). Toxicological profile for pentachlorophenol (update)(draft). Public Health Service, U. S. Department of Health and Human Services, Atlanta, G. A. 1999.
- Amman, R., Ludwig W. and K. H. Schleifer. 1995. Phylogenetic identification and *in-situ* detection of individual microbial cells without cultivation. *Microbiological Reviews*. 59: 143-169.
- Antoniou P., Hamilton J., Koopman B., Jain R., Holloway B., Lyberatos G. and Svoronos S. A. 1990. Effect of temperature and pH on the effective maximum specific growth rate of nitrifying bacteria. *Water Research*. 24: 97-101.
- Arcand Y., Hawari J. and Guiott S. 1995. Solubility of pentachlorophenol in aqueous solutions: the pH effect. *Water Research*. 29: 131-136.
- Barnes H.M. 2002. Wood preservation. *Encyclopedia of Pest Management*. Marcel Dekker, Inc.
- Bharathi S. and Vasudevan N. 2001. Utilization of petroleum hydrocarbons by *Pseudomonas fluorescens* isolated from petroleum contaminated soil. *Environmental International*. 26: 413-416.
- Birke V., Burmeier H. and Rosenau D. 2003. Design, construction and operation of tailored permeable reactive barriers. *Practice Periodical of Hazardous Toxic and Radioactive Waste Management*. 7: 264-280.
- Borazjani H., Diehl S., Britto R. and Lybrand M. 2005. *In-situ* biosparging of pentachlorophenol (PCP) contaminated groundwater. *Proceedings of Environmental Science and Technology*, 2005.
- Bustin S. A. 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *Journal of Molecular Endocrinology*. 29: 23-39.
- Cai M. and Xun L., 2002. Organization and regulation of pentachlorophenol degrading genes in *Sphingobium chlorophenolicum* ATCC 39723. *Journal of Bacteriology*. 184: 4672-4680.

- Cedar F. 1984. Historical and current developments of antisapstain chemical use. In Symposium on Preservatives in Wood Industry. Vancouver, Canada: University of British Columbia.
- CERCLIS. 2012. Comprehensive, Environmental, Response, Compensation, and Liability Information System public access database. <http://cumulis.epa.gov/supercpad/cursites/srchsites.cfm>.
- Chanama M. and Chanama S. 2011. Expression of pentachlorophenol degradative genes of *Sphingobium chlorophenolica* ATCC 39723 in *Escherichia coli*. Asia Journal of Public Health. 2: 78-83.
- Chandra R., Ghosh A., Jain R. K. and Singh S. 2006. Isolation and characterization of two potential pentachlorophenol degrading aerobic bacteria from pulp paper effluent sludge. Journal of General Applied Microbiology. 52: 125–130.
- Chen L., Maloney K., Krol E., Zhu B. and Yang J. 2009. Cloning, overexpression, purification, and characterization of the maleylacetate reductase from *Sphingobium chlorophenolicum* Strain ATCC 53874. Current Microbiology. 58:599–603.
- Chen L. and Yang J. 2008. Biochemical characterization of the tetrachlorobenzoquinone reductase involved in the biodegradation of pentachlorophenol. International Journal of Molecular Science. 9: 198-212.
- Cho H., Lee J. and Choi Y. 2003. The genetic diversity analysis of the bacterial community in groundwater by denaturing gradient gel electrophoresis (DGGE). Journal of Microbiology. 41: 327-334.
- Chu W. and Chan K. H. 2003. The mechanism of the surfactant aided soil washing system for hydrophobic and partial hydrophobic organisms. The Science of the Total Environment. 307: 83-92.
- Cline R. E., Hill R. H. J. and Phillips D. L. 1989. Pentachlorophenol measurements in body fluids of people in log homes and workplaces. Archives of Environment Contamination and Toxicology. 18: 475-481.
- Conesa H. M., Evangelou M. W. H., Robinson B. H., and Schulin R. 2011. A critical view of current state of phytotechnologies to remediate soils. Still a promising tool? The Scientific World Journal. 2012: 1-10.
- Cooper G. and Jones S. 2008. Pentachlorophenol and cancer risk: Focusing the lens on specific chlorophenols and contaminants. Environment Health Perspective. 116: 1001–1008.
- Copley S. D. 2000. Evolution of a metabolic pathway for degradation of a toxic xenobiotic: the patchwork approach. Trends in Biochemical Sciences. 25.

- Crawford R., Jung C. and Strap J. 2007. The recent evolution of pentachlorophenol (PCP)-4-monooxygenase (*pcpB*) and associated pathways for bacterial degradation of PCP. *Biodegradation*. 18:525–539.
- Crosby D.G. 1981. Environmental chemistry of pentachlorophenol. *Pure and Applied Chemistry*. 53: 1051-1080.
- Dai, M., Rogers. J. B, Warner J. R., and Copley S. D. 2003. A previously unrecognized step in pentachlorophenol degradation in *Sphingobium chlorophenolicum* is catalyzed by tetrachlorobenzoquinone reductase (PcpD). *Journal of Bacteriology*. 185: 302-310.
- Davis A., Campbell J., Gilbert C., Ruby M. V., Benett M. and Tobin S. 1994. Attenuation and biodegradation of chlorophenols in groundwater at a former wood treating facility. *Groundwater*. 32: 248-257.
- Demers P.A., Davies H. W., Friesen M. C., Hertzman C., Ostry A., Hershler R, and Teschke K. 2006. Cancer and occupational exposure to pentachlorophenol and tetrachlorophenol. *Cancer Causes Control*. 17:749-758.
- Dougherty R. 1978. Human exposure to Pentachlorophenol. *Pentachlorophenol: chemistry, pharmacology and environmental toxicology*, pp. 351-361. New York: Plenum Press.
- Federal Register, National primary and secondary drinking water regulations. Federal Register 54, 1999.
- Federal Remediation Technologies Roundtable (FRTR). 1995. Ex-situ Biological treatments: Composting. <http://www.frtr.gov/matrix2/section4/4-12.html>
- Fisher B. 1991. Pentachlorophenol: Toxicology and Environmental Fate. *Journal of Pesticide Reform*. 11: 2-5.
- Freeman M. H., Shupe T. F., Vlosky R. P. and Barnes H. M. 2003. Past, present and future of the wood preservation industry. *Forest Products Journal*. 53 : 8-15.
- Frey J., Angert E., and Pell A. 2006. Assessment of biases associated with profiling simple, model communities using terminal-restriction fragment length polymorphism based analyses. *Journal of Microbiological Methods*. 67: 9-19.
- Gary R. E., Gilliland R. D. and Smith E. E. 1985. Pentachlorophenol intoxication: Report of fatal case, with comments on clinical course and pathogenic anatomy. *Archives of Environmental Health*. 40: 161-164.
- Gerardi M. H. 2006. Wastewater bacteria. John Wiley and sons, inc., publication. 27-28.

- Gestel K. V., Mergaert J., Swings J., Coosemans J. and Ryckeboer J. 2003. Bioremediation of diesel oil-contaminated soil by composting with biowaste. *Environmental Pollution*. 125: 361-368.
- Griebler C. and Leuders T. 2009. Microbial biodiversity in groundwater ecosystems. *Freshwater Biology*. 54: 649-677.
- Groundwater Remediation Technologies Analysis Center. 1996d. Phytoremediation. publication # GWRTAC-T0-96-03. Pittsburgh, PA.
- Hambrick G. A., DeLaune R. D. and Patrick Jr. W. H. 1980. Effect of estuarine sediment pH and oxidation-reduction potential on microbial hydrocarbon degradation. *Applied and Environmental Microbiology*. 40: 365-369.
- Hattemer-Frey H. A. and Travis C. C. 1989. Pentachlorophenol: environmental partitioning and human exposure. *Archives of Environmental Contamination and Toxicology*. 18: 482-489.
- He L., Guangwei L., Xiufen L. and Chen J. 2008. Molecular characterization of bacterial community in aerobic granular sludge stressed by pentachlorophenol. *Journal of Environmental Sciences*. 20: 1243–1249.
- Hill T., Walsh K. A., Harris J. A., and Moffett B. F. 2003. Using ecological diversity measures with bacterial communities. *FEMS Microbial Ecology*. 43: 1-11.
- Jogler M. Siemens H., Chen H., Bunk B., Sikorski J. and Overmann J. 2011. Identification and targeted cultivation of abundant novel freshwater sphingomonads and analysis of their population substructure. *Applied and Environmental Microbiology*. 77: 7355–7364.
- Kalman D. A. and Horstman S. W. 1983. Persistence of tetrachlorophenol and pentachlorophenol in exposed wood workers. *Journal of Toxicology- Clinical Toxicology*. 20: 343-352.
- Kao C.M., Chaib C. T., Liub J. K., Yeh T. Y., Chena K.F. and Chend S. C. 2004. Evaluation of natural and enhanced PCP biodegradation at a former pesticide Manufacturing plant. *Water Research*. 38: 663–672.
- Kao C.M., Chen C. Y., Chen S.C., Chen H. Y. and Chen Y. L. 2008. Application of *in-situ* biosparging to remediate a petroleum-hydrocarbon spill site: Field and microbial evaluation. *Chemosphere*. 70: 1492-1499.
- Karn S. K., Chakrabarty S. K. and Reddy M. S. 2010 (a). Characterization of pentachlorophenol degrading *Bacillus* strains from secondary pulp-and-paper-industry sludge. *International Biodeterioration and Biodegradation*. 64: 609-613.

- Karn S. K., Chakrabarty S. K. and Reddy M. S. 2010 (b). Pentachlorophenol degradation by *Pseudomonas stutzeri* CL7 in the secondary sludge of pulp and paper mill. *Journal of Environmental Sciences*. 22: 1608-1612.
- Khan F. I., Husain T. and Hejazi R. 2004. An overview and analysis of site remediation technologies. *Journal of Environmental Management*. 71: 95-122.
- Langwaldt J. H., Mannisto M. K., Wichmann R. and Puhakka J. A. 1998. Simulation of *in-situ* subsurface biodegradation of polychlorophenols in air-lift percolators. *Applied Microbiology and Biotechnology*. 49: 663-668.
- Lee C., Guo Y., Kuei C., Chang H., Hsu J., Wang S., and Lao P. 2006. Human PCDD/PCDF levels near a pentachlorophenol contaminated site in Taiwan. *Chemosphere*. 65: 436-448.
- Lee P. K. H., Macbeth T. W., Sorenson K. S. Jr., Deeb R. A. and Varez-Cohen L. 2008. Quantifying genes and transcripts to assess the *in situ* physiology of “*Dehalococcoides*” sp. in a trichloroethane contaminated groundwater site. *Applied Environmental Microbiology*. 74: 2728-2739.
- Litchfield C. 2005. Thirty Years and Counting: Bioremediation in its Prime? *BioScience*. 55: 273-279.
- Louie T. M., Webster C. M. and Xun L. 2002. Genetic and biochemical characterization of a 2,4,6-trichlorophenol degradation pathway in *Ralstonia eutropha* JMP134. *Journal of Bacteriology*. 184: 3492-3500.
- Lowe S. E., Jain M. K. and Zeikus J. G. 1993. Biology, ecology, and biotechnological applications of anaerobic bacteria adapted to environmental stresses in temperature, pH, salinity, or substrates. *Microbiological reviews*. 57: 451-509.
- Macrae, A. 2000. The use of 16S rDNA methods in soil microbial ecology. *Brazilian Journal of Microbiology*. 31:77-82.
- Magurran, A. E. 2004. Measuring biological diversity. Oxford: Blackwell Publishing. The standard reference for conceptual and quantitative aspects of diversity measurement.
- McAllister, K. A., Lee H. and Trevors J. 1996. Microbial degradation of pentachlorophenol. *Biodegradation*. 7: 1-40.
- McLean, D., Eng A., Walls C., Dryson E., Harawira J., Cheng S., Wong K. C., Manneje A, Gray M., Shoemack P., Smith A. and Pearce N.. 2008. Serum dioxin levels in former New Zealand sawmill workers twenty years after exposure to pentachlorophenol ceased. *Chemosphere*. 74: 962-967.

- Noven K. 1988. Changes in levels of organochloride pesticides, polychlorinated biphenyls, dibenzo-p-dioxins and dibenzo-furans in human milk from Stockholm. 1972-1985. *Chemosphere*. 17: 39-49.
- Nikodem P., Hecht V., Schlomann M. and Pieper D. H. 2003. New bacterial pathway for 4- and 5-chlorosalicylate degradation via 4-chlorocatechol and maleylacetate in *Pseudomonas* sp. strain MT1. *Journal of Bacteriology*. 185: 6790–6800.
- O’Sullivan L. A. and Mahenthiralingam E. 2005. Biotechnological potential within the Genus *Burkholderia*. *Letters in Applied Microbiology*. 41: 8-11.
- Ryan J. E. 1985. Chlorinated dibenzo-p-dioxins and chlorinated dibenzofurans in Canadian human adipose tissue. *Chemosphere*. 14: 697-706.
- Sánchez M. A. and González B. 2007. Genetic characterization of 2,4,6-trichlorophenol degradation in *Cupriavidus necator* JMP134. *Applied and Environmental Microbiology*. 73: 2769-2776.
- Schmittgen T. D. and Livak K. J. 2008. Analyzing real-time PCR data by the comparative C_T method. *Nature Protocols*. 3: 1101-1108.
- Sharma A., Thakur I. S. and Dureja P. 2009. Enrichment, isolation and characterization of pentachlorophenol degrading bacterium *Acinetobacter* sp. ISTPCP-3 from effluent discharge site. *Biodegradation*. 20: 643-650.
- Shen D. S., Liu X. W. and Feng H. J. 2005. Effect of easily degradable substrate on anaerobic degradation of pentachlorophenol in an up flow anaerobic sludge blanket (UASB) reactor. *Journal of Hazardous Materials*. B119: 239–243.
- Slater J. H. and Lovatt D. 1984. Biodegradation and the significance of microbial communities, p. 439-485. In I. Gibson and T. David (ed.), *Microbial degradation of organic compounds*. Marcel Dekker, Inc., New York.
- Smith C. J. and Osborn A. M. 2008. Advantages and limitations of quantitative PCR (Q-PCR) based approaches in microbial ecology. *Federation of European Microbiological Societies Microbial Ecology*. 67: 6-20.
- Stokes C. E. 2011. Effects of in-situ biosparging on pentachlorophenol (PCP) degradation and bacterial communities in PCP contaminated groundwater. PhD dissertation. Mississippi State University. 2: 27-28.
- Stults J. R., Snoeyebos- West O., Methe B., Lovely D. R. and Chandler D. P. 2001. Application of 5’ fluorogenic exonuclease assay (*TaqMan*) for quantitative ribosomal DNA and rRNA analysis in sediments. *Applied Environmental Microbiology*. 67: 2781-2789.
- Structure of pentachlorophenol: <http://en.wikipedia.org/wiki/Pentachlorophenol>.

- Tirola M.A., Wang H., Paulin L., and Kulomaa M.S. 2002. Evidence for natural horizontal transfer of the *pcpB* gene in evolution of polychlorophenol-degrading sphingomonads. *Applied Environmental Microbiology*. 68: 4495-4501.
- Tondeur Y., Nestrick T., Silva H. A., Vining B. and Hart J. 2010. Analytical procedures for the determination of polychlorinated-p-dioxins, polychlorinated dibenzofurans, and hexachlorobenzene in pentachlorophenol. *Chemosphere*. 80: 157-164.
- Treusch A. H., Leininger S., Kletzin A., Schuster S. C., Klenk H. P. and Schleper C. 2005. Novel genes for nitrite reductase and amonorelated proteins indicate a role of uncultured mesophilic *crenarchaeota* in nitrogen cycling. *Environmental Microbiology*. 7: 1985-1995.
- U. S. Environmental Protection Agency. 1978. Pentachlorophenol position document 1. Washington, DC: Office of Pesticides and Toxic Substances.
- U. S. Environmental Protection Agency. 1987. Final determination and indent to cancel and deny applications for registrations of pesticide products containing pentachlorophenol (including but not limited to its salts and esters) for non-wood uses. US Environmental Protection Agency. *Federal Register*. 52: 2282-2293.
- U. S. Environmental Protection Agency. 1990. Suspended, cancelled and restricted pesticides. Washington, DC: Office of Pesticides and Toxic Substances.
- U. S. Environmental Protection Agency. 1996f. A citizen's guide to in-situ thermal desorption. Office of Solid Waste and Emergency Response, US Environmental Protection Agency. Publication # EPA 542-F-96-005, Washington, DC.
- U.S. Environmental Protection Agency. 1998b. Landfarming. Office of the Underground Storage Tank, US Environmental Protection Agency. Publication # EPA 510-B-95-007.
- U. S. Environmental Protection Agency. 1998f. Field application of in-situ remediation technologies: groundwater circulation wells. US EPA. Publication # EPA 542-R-98-009, Washington, DC.
- U. S. Environmental Protection Agency. 1999. Integrated Risk Information System (IRIS) on Pentachlorophenol. National Center for Environmental Assessment, Office of Research and Development, Washington, DC.
- U. S. Environmental Protection Agency. 2004. Environmental fate of pentachlorophenol. EPA-HQ-OPP-2004-0402-0014.
<http://pi.ace.orst.edu/search/getDocketDocument.s?document=EPA-HQ-OPP-2004-0402-0014>

- U. S. Environmental Protection Agency. 2008. Reregistration eligibility decision for pentachlorophenol. EPA 739-R-08-008.94 pp.
- Vallecillo A., Garcia-Encina P. A. and Pena M. 1999. Anaerobic biodegradability and toxicity of chlorophenols. *Water Science and Technology*. 40: 161-168.
- Vander Zande A. 2010. Exploration of management options for pentachlorophenol (PCP) paper for the 8th meeting of the UNECE CLRTAP Task Force on Persistent Organic Pollutants, Montreal, 18-20, May 2010.
- Vidali M. 2001. Bioremediation: An Overview. *Pure and Applied Chemistry*. 73:1163-1172.
- Wang H., Marjomaki V., Ovod V., and Kulomaa M. S. 2002. Subcellular localization of pentachlorophenol-4-monooxygenase in *Sphingobium chlorophenolicum* ATCC 39723. *Biochemical and Biophysical Research Communication*. 299: 703-709.
- Wang X., Meier R. J., and Wolfbeis O. S. 2012. Fluorescent pH-sensitive nanoparticles in an agarose matrix for imaging of bacterial growth and metabolism. *Angewandte Chemie International Edition*. 51: 1-5.
- Warren J. S., Lamparski L. L., Johnson R. L. and Gooch R. M. 1982. Determination of pentachlorophenol volatilized from wood via collection on silica gel. *Bulletin of environmental contamination and toxicology*. 29: 719-726.
- Watt B. and Brown F. V. 1985. Effect of the growth of anaerobic bacteria on the surface pH of solid media. *Journal of clinical pathology*. 38: 565-569.
- Wijngaard A. J. V., Kleij R. G. V., Doornweerd R. E. and Janssen D. B. Influence of organic nutrients and co-cultures on the competitive behavior of 1,2-dichloroethane-degrading bacteria. 1993. *Applied and Environmental Microbiology*. 59: 3400-3405.
- Xun L. and Gisi M. R. 2003. Characterization of chlorophenol-4-monooxygenase (tftD) and NADH: flavin adenine dinucleotide oxidoreductase (tftC) of *Burkholderia cepacia* AC 1100. *Journal of Bacteriology*. 185: 2786-2792.
- Yang C. F., Lee C. M. and Wang C. C. 2006. Isolation and physiological characterization of the pentachlorophenol degrading bacterium *Sphingomonas chlorophenolica*. *Chemosphere*. 62: 709-714.